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Nutritional Value of Modified Novel Oilseed Meals in Teleost Diets

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NUTRITIONAL VALUE OF MODIFIED NOVEL OILSEED MEALS IN TELEOST
DIETS

BY
TOM KASIGA

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Wildlife and Fisheries Sciences

South Dakota State University

2018

NUTRITIONAL VALUE OF MODIFIED NOVEL OILSEED MEALS IN TELEOST
DIETS

TOM KASIGA

This dissertation is approved as a credible and independent investigation by a candidate for the Doctor of Philosophy in Wildlife and Fisheries Sciences degree and is acceptable for meeting the dissertation requirements for the degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this research to my late parents. My father wanted me to be a medical doctor but Doctor of Philosophy would not have been a disappointment to him.

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LIST OF ABBREVIATIONS

μL: Microliters

μmoles: Micromoles

♀: Female

♂: Male

Abs: Absorbance

AC: Aerobic conversion

ACCM: Aerobically converted carinata meal

ACH: Alternative complement hemolysis

ADC: Apparent digestibility coefficient

AM: Ante meridiem

ANF: Antinutritional factor

ANOVA: Ananalysis of variance

ANPU: Apparent net protein utilization

AOAC: Association of Official Analytical Chemists

APD: Apparent protein deposition

ARA: Arachidonic acid

Arg: Arginine

B: Bone marrow

BAPNA: *N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride

BCA: Bicinchonic acid

C: Celsius

C: Complement protein

Ca: Calcium

CaCl₂: Calcium chloride

Cat. No: Catalogue number

CEAA: Conditionally-essential amino acid

CM: Carinata meal

cm: Centimeter

CMC: Carboxymethyl cellulose

CN: Cranial nerve

CP: Cold pressed

CPAC: Cold pressed and aerobically converted

CPC: Corn protein concentrate

CPCM: Cold pressed carinata meal

CPEX: Cold pressed and extruded

CPEXAC: Cold pressed, extruded and aerobically converted

CPEXSE: Cold pressed, extruded and solvent extracted

CPEXSEAC: Cold pressed, extruded, solvent extracted and aerobically converted

CPSE: Cold pressed and solvent extracted

CPSEAC: Cold pressed, solvent extracted and aerobically converted

Cr₂O₃: Chromic oxide

Cu: Copper

DHA: Docosaheptaenoic acid

DI: Deiodinase activity I

DII: Deiodinase activity II

DIII: Deiodinase activity III

dL: Deciliter

EAA: Esseential amino acid

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

EX: Extruded

FAO: Food and Agriculture Organization of the United Nations

FCR: Feed conversion ratio

Fe: Iron

FM: Fish meal

FMO3: Flavin-containing monooxygenase 3 gene

g: gram

g: gravitation force

GE: Gross energy

GIT: Gastrointestinal tract

GLS: Glucosinolate

H₂O: Water

Hb: Hemoglobin

HCl: Hydrochloric acid

HE: Hexane extracted

His: Histidine

Hk: Hematocrit

HP: Horsepower

HPLC: High performance liquid chromatography

hrp: Horseradish peroxidase

HSB: Hybrid Striped Bass

HSD: Honest significance difference

HSI: Hepatosomatic index

HUFA: Highly unsaturated fatty acid

Hz: Hertz

IACUC: Institutional Animal Care Use Committee

Ile: Isoleucine

Inc: Incorporated

IRD: Inner ring deiodination

ITC: Isothiocyanates

K: Fulton's condition factor

K: Potassium

$\text{K}_3\text{Fe}(\text{CN})_6$: Potassium ferricyanide

Kcal: Kilocalorie

KCN: Potassium cyanide

Kg: Kilogram

KW: Kilowatt

L: Liter

Leu: Leucine

LLC: Limited Liability Company

ln: Natural log

Lys: Lysine

M: Molar

MAC: Membrane attack complex

MCHC: Mean corpuscular hemoglobin content

Met: Methionine

Mg: Magnesium

mg: Milligram

MgCl₂: Magnesium chloride

mins: Minutes

MJ: Millijoule

mL: Milliliter

mm: Millimeter

mM: Millimoles

MMT: Million metric tons

Mn: Manganese

N: Normality

Na: Sodium

Na₂B₄O₇: Sodium borate

NASS: National Agricultural Statistics Service

NC-IUB: Nomenclature Committee of the International Union of Biochemistry

NFE: Nitrogen free extract

ng: Nanogram

nm: Nanometer

nmoles: Nanomoles

Nod: Nucleotide-binding oligomerization domain

NRC: National Research Council

NSP: Nonstarch polysaccharide

OD: Optical density

ORD: Outer ring deiodination

P: Phosphorous

p: probability value

PBS: Phosphate buffered saline

PDA: PhotoDiode Array

PEPt: peptide transporter

PER: Protein efficiency ratio

PGBP: Peptidoglycan-binding proteins

pH: Potential of hydrogen

Phe: Phenylalanine

PM: Post meridiem

RAS: Recirculating aquaculture system

RBC: Red blood cell

RBT: Rainbow Trout

Ref: Reference

ROS: reactive oxygen species

rpm: Revolutions per minute

RSM: Rapeseed meal

S: Sulfur

SBM: Soybean meal

SD: Standard deviation

SE: Solvent extraction

SE: Standard error

SECM: Solvent extracted carinata meal

SE-SBM: Solvent extracted soybean meal

SGR: Specific growth rate

SSI: Spleen somatic index

T: Thymus

T₂: Diiodothyronine

T₃: Triiodothyronine

T₄: Thyroxine

Thr: Threonine

TIU: Trypsin inhibitor unit

TLR: Toll-like receptor

TMA: Trimethylamine

TMAO: Trimethylamine oxide

TMB: Trimethylbenzidine

Tris-HCl: Hydroxymethyl amino methane-hydrochloric acid

Trp: Tryptophan

U: Units

UDP: Uridine diphosphate glucose

UN: United Nations

UPLC: Ultra performance liquid chromatography

USDA ARS: United States Department of Agriculture Agricultural Research Service

USDA: United States Department of Agriculture

Val: Valine

VFD: Variable frequency drive

VFSI: Visceral fat somatic index

VSI: Viscerosomatic index

WCM: Double-washed carinata meal

YSI: Yellow springs instrument

Zn: Zinc

ABSTRACT

NUTRITIONAL VALUE OF MODIFIED NOVEL OILSEED MEALS IN TELEOST

DIETS

TOM KASIGA

2018

Carinata *Brassica carinata* and camelina *Camelina sativa* are being genetically modified to improve their composition for biofuel production and other agricultural applications. After oil extraction, the residual de-oiled meals (<1% oil) are high (>40%) in protein. However, their inclusion in animal feeds is limited to 10% because of high levels of antinutritional factors (ANFs), mainly glucosinolates (GLS), sinapine, and crude fiber that limit diet intake, nutrient utilization, and lower thyroxine production, resulting in reduced growth.

The nutritional value of carinata and camelina seed meals to fish were assessed by subjecting cold pressed (CP) carinata and camelina meals to extrusion (EX), solvent extraction (SE) and aerobic conversion (AC) or sequential process combinations to determine which process(es) yielded improved composition. Carinata meals generally yielded more crude protein and lower fiber. The primary protein increase for both meal types was due to oil removal and AC. Fiber increased with AC, but that step reduced GLS by at least 70%. Palatability was generally improved by SE. Apparent digestibility coefficients (ADCs) for protein were generally higher in carinata than camelina meals for both Rainbow Trout *Oncorhynchus mykiss* (RBT) and Hybrid Striped (Sunshine) Bass *Morone chrysops* ♀ x *M. saxatilis* ♂ (HSB). Overall protein ADCs were higher in HSB than in RBT. The use of AC on protein ADC was more effective in camelina meals.

Two experiments were conducted to determine the maximum GLS concentration from cold-pressed carinata meal (CPCM) containing 61.2 μ moles of GLS and 6.07 mg of sinapine/g of meal that could be tolerated in HSB diets. In experiment 1, there was no effect of up to 2.71 μ moles of GLS and 0.181 mg of sinapine/g of diet on diet palatability, thyroxines production, deiodinase enzyme activity, and consequently HSB growth. In experiment 2, concentrations of ≥ 5.58 μ moles of GLS and ≥ 0.54 mg of sinapine/g of diet reduced feed consumption, utilization, and growth. Reduction in palatability due to GLS and sinapine from CPCM may not enable fish to consume enough GLS and / or GLS breakdown products to impair thyroid function. GLS were not lethal to HSB. Based on meal composition, protein ADCs, palatability and GLS tolerance, solvent extracted aerobically converted carinata meal, followed by a single wash (ACCM), was chosen for growth performance trials.

In the RBT growth trial, we determined how much fish meal (FM) could be replaced with ACCM. We replaced 25, 50 and 75% of FM in the reference diet containing 20% FM as the sole animal meal, composing diets of 44% crude protein and 17% crude lipid. Diets were balanced for nutrients and fiber. After a 56-day growth period, replacement of more than 25% FM by ACCM resulted in reduced growth partly due to reduced feed consumption. Condition factor (Fulton's K) decreased with increased FM replacement. Feed conversion ratio (FCR) had an inverse relationship with diet consumption. Results of this study showed that more than 25% of FM cannot be replaced by ACCM in low FM/animal (20%) diets of RBT; improved utilization of ACCM by RBT may occur with more animal meal inclusion or additional processing of ACCM to improve the feeding value.

In the HSB growth trial, we determined the maximum inclusion of ACCM or double-washed carinata meal, without AC (WCM) in low (20%) animal diets. We included ACCM at 10 and 30%; and WCM at only 30% of the diets. All diets contained ~44% crude protein 12% crude lipid, and were balanced for fiber. After a 106-day growth period, we observed that HSB fed 30% WCM had a similar weight gain to HSB fed the FM reference diet and 30% ACCM but better than HSB fed 10% ACCM. High (30%) amounts of ACCM or WCM improved feed consumption. FCR of WCM was better than that of ACCM. HSB fed 30% WCM had smaller livers and higher condition factors than HSB fed other treatment diets. Survival (>99%) was similar among treatments. Hematocrit (Hk) and hemoglobin (Hb) contents of HSB were improved by ACCM but not WCM. These results showed that the second wash in WCM improved feed utilization more than ACCM. However, the extra wash step in WCM likely reduced the iron content of WCM resulting in lower Hk and Hb.

At the end of the RBT growth trial, trypsin activity, protein ADCs, amino acid ADCs and availability were measured to account for observed differences in protein utilization and thus growth. Trypsin activity and protein ADCs were not altered with increasing ACCM in diets. Replacement of more than 25% of FM reduced apparent digestibility of arginine, histidine, isoleucine, leucine, phenylalanine, threonine, valine, and tyrosine. However, the above essential amino acids (EAAs) were not decreased in serum due to FM replacement with ACCM, although serum from RBT fed 75% ACCM contained lower lysine concentrations. ACCM diets had lower EAA peak concentrations and a slower release of EAAs in serum. Cumulative total EAAs in serum also decreased with ACCM inclusion. The pattern of total EAAs in serum for most sampling intervals

best associated with muscle EAAs for the reference and 10 and 15% ACCM. Ratios of EAAs to lysine showed that tryptophan was the most limiting EAA. However, isoleucine, leucine, methionine, and phenylalanine were also inadequate for muscle synthesis for the first 9-12 hours after force-feeding. Optimal time for muscle synthesis was 36 or more hours because all EAAs were adequate except for isoleucine in the muscle of RBT fed the 10% ACCM diet.

Following the HSB growth trial, trypsin activity, protein ADCs, amino acid ADCs and availability were measured to explain the observed differences in protein utilization and thus growth. Inclusion of up to 30% processed CM did not alter trypsin activity or protein ADCs. However, 30% ACCM reduced isoleucine, leucine, phenylalanine, threonine and valine ADCs. Feeding high ACCM (30%) reduced serum arginine and leucine. All inclusions of ACCM or WCM increased serum methionine. High inclusions of ACCM or WCM (30%) increased serum tryptophan and valine. The reference and 30% WCM diets resulted in the highest total essential amino acids (EAAs) in serum but the release of total EAAs in serum of HSB fed 30% WCM was elevated continuously over a longer period. High inclusions of ACCM or WCM (30%) increased muscle histidine but resulted in lower leucine and phenylalanine. Only 30% WCM increased muscle lysine and valine. However, all inclusion levels of ACCM or WCM increased muscle methionine. High inclusions of ACCM or WCM (30%) in diets resulted in more available total EAAs over a longer period. Muscle EAA to lysine ratios showed that only histidine concentrations were adequate for muscle synthesis over the 36-hour period.

CHAPTER 1

INTRODUCTION AND BACKGROUND OF THE STUDY

Food fish demand increased by 3.2% from 1961-2013, double the rate of human population growth of 1.6% in the same period. In addition, per capita fish consumption increased from 9.9 kgs in 1960s to 20.1 kgs in 2014. Total fish production from wild capture fisheries from the mid-1980s has remained relatively constant and the abundance of many high value species are in decline. Over the last few decades, fish production from commercial aquaculture rapidly grew, increasing the contribution of aquaculture to total fish production from about 9.3% in 1985 to 44.1% in 2014 to satisfy the increased food fish demand. This has increased the percentage of fish produced by aquaculture consumed by humans from <10% in 1960s to 50% in 2014 (FAO 2016). United States of America (USA) was ranked 17th among the top 25 fish producing countries, with its production of 425.9 tons, representing 0.42% of the global aquaculture production in 2014 (FAO 2016). Channel Catfish *Ictalurus punctatus* is the predominant finfish farmed in United States (USDA NASS 2014, FAO 2018) followed by Rainbow Trout *Oncorhynchus mykiss* (RBT), Atlantic Salmon *Salmo salar*, and Tilapia genera. The combined production of Striped and Hybrid Striped Bass (HSB) in 2016 was about 10.4% of Channel Catfish production (FAO 2018).

Fish are an excellent source of protein and micronutrients for a balanced human nutrition required to maintain good health. Fish accounted for 17% and 6.7% of animal and total protein respectively, consumed by humans in 2013 (FAO 2016). Further, there is an increased dietary need for a high quality protein especially in the developing countries when compared to the developed countries such as USA and the European

Union. However, most fish consumed in the developed countries is imported from developing countries (FAO 2016). The current human population is ~7.6 billion and is projected to reach 8.6 billion in 2030 and 9.8 billion in 2050 (UN 2017). However, fish production from capture fisheries has stagnated for the last three decades (FAO 2016). Therefore, aquaculture will be required to provide an estimated additional 20.1 MMT and 44.2 MMT of food fish by 2030 and 2050 respectively, at the current (2014) per capita consumption of 20.1 kg. Fish feed production will have to be increased by at least 20.1 MMT and 44.2 MMT, respectively, to support such an increase in food fish production. The percentage of fed fish has also been increasing with increase in aquaculture production from 59.9% in 2008 (FAO 2012) to 69.2% in 2014 (FAO 2016) and will likely increase with more aquaculture production.

Fish meal (FM) has traditionally been used as the main protein source in fish feeds especially for carnivorous fishes and is also used in feed production for other animal production sectors. The global production of FM decreased from 4.3 MMT in 2005 to 3.7 MMT in 2008 and is projected to approach to 3.5 MMT by 2020. Similarly, use of FM in fish feeds reduced from 18.7% in 2005 to 12.8% in 2008 and is projected to reduce further to 4.9% by 2020 (Tacon et al. 2011). It is becoming increasingly challenging for fish feed manufacturers to use FM in fish feed production to meet the increasing demand of fish feed. This is because of the difficulty in sourcing FM and the associated costs (Naylor et al. 2009). Therefore, there is a need to look for suitable alternatives that can both meet the nutritional requirements of fish and are environmentally friendly (Gatlin et al. 2007).

A major focus of fish nutrition research, for about the last two decades, has been to investigate protein alternatives to FM by either partially or fully substituting FM in fish feeds (Hardy 1999; Lim et al. 2008). Animal and plant products and their by-products that have been tested to partially or fully substitute FM in fish feeds include blood meal (Allan et al. 2000), meat and bone meal (Bureau et al. 2000), poultry feather meal (Bureau 2000), poultry by-product meal (Yigit et al. 2006), fish processing waste (Sotolu 2009), soybean meal (Zhou 2005), cottonseed meal (El-Saidy et al. 2004), distillers' by-products (Cheng and Hardy 2004; Thompson et al. 2008) and sunflower cake (Sanz et al. 1994). The above protein sources for fish feeds differ in protein content, amino acid composition, minerals and antinutrients (NRC 2011) which determines the inclusion levels and/ or the percentage of FM that can be replaced in fish feeds. To be a viable candidate for FM replacement, the feedstuff should be widely available, be produced at a competitive price, and should be easy to handle, store, ship and use in feed production. The feedstuff should also possess the required nutritional characteristics such as low fiber, starch, antinutrients and conversely, should be relatively high in protein content and the required amino acids, and have high nutrient digestibility and palatability (Gatlin et al. 2007).

Most plant meals and their co-or by-products may be more suitable for use in large-scale fish feed production compared to use of FM or other animal products and co-or by-products because there is a wide variety of plant feedstuffs available that can be formulated and blended to counter nutritional deficiencies in one feedstuff or another (NRC 2011). However, there are factors that limit use of high levels of plant products in fish feeds and these include: 1) antinutritional factors (ANFs) such as protease inhibitors,

glucosinolates, saponins, gossypol, non-starch polysaccharides, tannins, phytic acid (Gatlin et al. 2007; Krogdahl et al. 2010) and sinapine (Burel et al. 2000c), which all need to be reduced to levels tolerated by fish; 2) low levels of some essential amino acids like lysine and methionine (Gatlin et al 2007); and, 3) high energy density due to the high carbohydrate content (starch) which is of little or no nutritional value to carnivorous fish (NRC 2011).

Soybean meal (SBM) is considered the best plant feedstuff for use in fish feeds because it is high in protein, has a balanced amino acid profile and it is highly digestible (Gatlin et al. 2007). However, fish feed production uses less than 5% of the global soybean meal production and the cost of SBM has increased mainly due to increased competition for SBM with other animal feed production sectors (Hardy 2010). This has prompted researchers in animal nutrition to look at alternative oil seed meals such as Camelina *Camelina sativa* and Carinata *Brassica carinata* meals.

Carinata originated from Ethiopia (Ethiopian mustard) and was introduced in USA in 1957 initially as an oil seed crop but later produced as a vegetable crop (Stephens et al. 1970). Carinata was also introduced in Canada as an oilseed crop (Rakow and Getinet 1998; Getinet et al. 1999) with potential for ethanol and biodiesel production or specialty fatty acids (Barro et al. 2011; Pan et al. 2012) because of its tolerance to biotic and abiotic stressors under semi-arid conditions compared to other oil seeds (Rakow 1995). Carinata seeds are large with large embryos and a lower proportion of hulls, which results in higher protein and lower fiber contents (Getinet et al. 1999). The protein content of carinata seeds ranges from 25.9 to 30.5% on a dry basis (Pan et al. 2012). Carinata seeds may be composed of up to 40% oil, with the highest composition of the oil

being erucic acid (Rakow and Getinet 1998). Carinata oil also contains 10.2-16% linolenic acid and 13.7-18.9% linoleic acid (Warwick et al. 2006).

Carinata just like any other plant meal contains ANFs (Ikedo et al. 1996).

Antinutritional factors are substances that directly or indirectly interfere with food utilization in living systems, causing negative effects on health and production of animals (Makkar 1993). Antinutritional factors alter feed taste and lower feed intake, interfere with feed utilization, and can cause abnormal changes in the gut structure (Francis et al. 2001). Carinata seed meal contains glucosinolates, phytates (Rakow and Getinet 1998; Pedroche et al. 2004), tannins (Francis et al. 2001), erucic acid (Warwick et al. 2006), sinapine (Matthaus and Angelini 2005), and trypsin inhibitors (Bell and Rakow 1996). The above ANFs make carinata seed meal less suitable for use in fish feeds (Gatlin 2007), requiring further processing to reduce or eliminate them. Most carinata seed meal is discarded as a waste due to high glucosinolate contents and when it is used as an animal feed ingredient, it is mixed with noug (thistle) and flax seed meals (Getinet et al. 1997).

Camelina was introduced in the Americas as a weed (false flax) in flax seed (Putnam et al. 1993). Camelina is historically known to have been favored for cultivation in central and southern USA because it requires low inputs, produces a high yield, it is frost and drought tolerant and it is also resistant to insect pests (Porcher 1863). Camelina has been recently explored as an oilseed crop because of its high oil content and fatty acid profile. Camelina seeds contain about 27.1 to 33.3% protein (Gugel and Falik 2006), 43% oil and the oil consists of 30-40% linolenic acid and 3% erucic acid (Zubr and Mathaus 2002). It is an oilseed crop whose oil has the potential of being a biofuel additive or used

directly as a biofuel (Johnson 2007). Camelina seed meal can be used as an ingredient in animal feeds because the meal contains about 40% protein (Pilgeram 2007) with a balanced amino acid profile (Bonjean and Le Goffic 1999). Camelina seed meal also contains glucosinolates, phytic acid, trypsin inhibitors (Budin et al. 1995) and tannins (Matthaus 1997), which need to be reduced or removed before it is used in fish feeds.

Some of the reported effects on fish of the ANFs in carinata and camelina seed meals include: restriction of enzymes reaching their target nutrients caused by fiber (Ahmad et al. 2004) because fish cannot digest fiber due to lack of microbially derived cellulase (De Silva and Anderson 1995); reduced growth, changes in thyroid histology and deiodinase enzymes in the liver, brain and kidney (glucosinolates, Burel et al. 2000a); having an astringent bitter flavor, binding digestive enzymes and complexing with nutrients like proteins, minerals (tannins, Sandoval and Carmona 1998); reduction of diet palatability due to a bitter taste (Sinapine, Naczek et al. 1998); reduction of protein digestibility (protease inhibitors, Krogh et al. 1994); reduction of mineral availability (phytic acid, Vielma et al. 2004); and lipidosis of the epicardial connective tissue (erucic acid, Hendricks 2002).

The ANFs in carinata and camelina seed meals can be reduced by one or a combination of the several processes involved in the preparation of the fish feed ingredients or the actual feed making. Phytates and tannins can be reduced by dehulling seeds (Francis et al. 2001) and fermentation with yeast or lactic acid bacteria (Mukhopadhyay and Ray 1999). Thorough oil removal (<1%) from carinata and camelina meals lowers the chances of any negative effects of erucic acid to fed animal. Fiber can be reduced by use of exogenous enzymes (Adeola and Cowieson 2011) and /or microbial

fermentation (Pandey et al. 2000). Heating can reduce trypsin inhibitors (Francis et al. 2001) and glucosinolates (Burel et al. 2000b). Glucosinolates (Makkar and Becker 1997) are also broken down if any meal processing involves water. Sinapine (Das et al. 2009) are water-soluble and can be extracted by water.

De-oiled (<1% oil) carinata and camelina meals are likely >40% in crude protein because of the high (>40%) oil contents of the seeds. Therefore, the seed meals could be good protein sources for use in fish feeds provided that antinutrients are reduced or removed. The protein contents of de-oiled carinata and camelina seed meals may further be improved by microbial processing with bacteria, fungi, or yeast. Microbes produce enzymes that fishes do not produce such as β -amylases, β -glucanases, β -mannases, β -xylanases that break down the β -glycosidic bonds in the carbohydrate molecules (Rust 2002) to release sugars that can be transformed into short chain fatty acids such acetic acid, propionic acid and butyric acid (Clements et al. 1994) that may provide energy to fish (Tocher 2003). Utilization of nonstarch polysaccharides (NSP) and soluble sugars by microbes concentrates the remaining protein. Microbes themselves also contribute to the protein content (single-cell protein) of the fermented product. In addition, the amino and fatty acid composition of the microbes used for a given process in the overall fermentation process can increase the essential amino and fatty acids in the fermented meals as opposed to the raw meals.

The breeding goal of oilseed geneticists has been to come up with a carinata strain with a higher oil and glucosinolate content that can provide germ plasm for high erucic acid and other industrial oils and the high allyl glucosinolate (sinigrin) meal to be used as a bio pesticide (Taylor et al. 2010). Therefore, farmers in the more arid areas of the

Dakotas are being encouraged to integrate carinata into their crop rotation systems.

Agragen (Cincinnati, OH) is genetically modifying camelina in partnership with Great Plains Oil and Exploration, LLC (Cincinnati, OH) with the aim of improving desirable traits such as oil content, seed yield, viability in expanded environments, and resistance to disease, weeds and pests (Moser 2010).

With oil extraction, the remaining carinata and camelina seed meals will be available for use in the animal feed industry and the successful use of the two seed meals will depend on how well antinutrients will be removed from the seed meals. If carinata and camelina seed meals are to be used to replace much of FM in carnivorous fish feeds, increasing protein contents will reduce their inclusion levels when replacing FM on an equal protein basis.

The additional advantage of using plant meals in fish feeds that are unfermented or fermented is that β -glucans in the cell walls of plants; and fungi and bacteria (Sealey and Gatlin 2001; Gatlin 2002) used in fermentation can elicit an immune response in fish. Lipopolysaccharides (Erridge et al. 2002) and peptidoglycans (Madigan et al. 1997) in the cell walls of bacteria used for fermentation can also elicit an immune response in multicellular organisms. Improving the health status of fish improves survival and general performance (NRC 2011).

Data reported on the use of carinata and camelina meals in fish diets is recent, with more research done on camelina than carinata meal. Hixson et al. (2016a) observed that Atlantic Cod *Gadus morhua* tolerated 24% of de-oiled camelina meal in a diet containing 41.7% FM. However, in another experiment, cod could not tolerate 15% de-oiled camelina meal in a diet containing 45% FM. Hixson et al. (2016b) also observed

that RBT and Atlantic Salmon could tolerate 14% and 8% respectively, of de-oiled camelina meal in diets without affecting fish growth. The diets used in the above studies contained 39 and 32% animal meals, respectively. Apparent digestibility coefficients for high oil residual, solvent extracted (SE) and toasted SE camelina meals in RBT reported by Fraser et al. (2017) were 90.6, 90.0 and 90.2% respectively. Anderson et al. (2018) replaced 28.6% of FM in a reference diet containing 35% FM and 45% total animal meals with soaked and heated (86°C for 10 mins) carinata and camelina meals (15% of the diet) in diets of RBT fingerlings. After 111 days of the study, they observed reduced growth with FM replacement with camelina but not with carinata meal. However, there was no difference in growth between RBT fed the carinata and camelina diets.

The above digestibility study of camelina meals in RBT shows that they are highly digestible in fish. However, both camelina and carinata meals reduced feed intake and nutrient utilization, resulting in low ($\leq 15\%$) tolerance yet diets contained high FM ($\geq 25\%$) and total animal ($\geq 35\%$) meals. Replacement of FM with carinata and camelina meals would require processing of the two meals to reduce ANFs that lower feed intake, digestibility, and nutrient utilization; as well as increase protein to reduce inclusion levels of both meals if they are to replace more FM on an equal protein basis. Therefore, there was need to develop new knowledge on how well fish would utilize nutrients from processed carinata and camelina meals.

Statement of the Problem

Carinata and camelina seeds are being genetically modified to improve their composition for biofuel production and other agricultural applications. After oil extractions, the de-oiled meals will be available for use in the animal feed industry. Fish

nutritionists have been searching for the recent two decades for alternative protein sources to fish meal (FM) for use in fish diets especially for carnivorous fish. This is because FM is no longer economical and sustainable for the highly expanding fish feed industry due to a rapidly expanding aquaculture industry. The high (>40%) protein contents of de-oiled (<1%) carinata and camelina meals make them ideal candidates for FM replacement in fish diets. Few studies report use of carinata and camelina meals as protein sources in fish diets and the data limits inclusion of carinata and camelina meals in fish diets to $\leq 15\%$. This is because most of the meals used in those studies are not processed beyond oil extraction to lower antinutrients such as glucosinolates, sinapine and crude fiber, that lower diet intake, nutrient utilization and thyroxine production, resulting in reduced growth.

Little knowledge is available on how processing carinata and camelina meals may improve meal composition, which may consequently improve meal palatability, digestibility, nutrient utilization, and growth performance. Therefore, we hypothesize that carinata and camelina seed meals processed beyond oil extraction to lower antinutrients and / or increase protein may replace more FM in fish diets on an equal protein basis.

Research Questions

Plant protein sources are being investigated as sustainable sources of proteins to replace FM in fish feeds but some questions still arise as to whether plant meals can fully substitute fish meal (FM) in carnivorous fish. The overall aim of this study was to answer the following questions:

1. What is the effect of processing carinata and camelina seed meals on meal composition?

2. How does the digestibility of nutrients and meal palatability differ among different processed carinata and camelina meals
3. What is the tolerance limit for glucosinolate and sinapine from carinata meals in fish?
4. How much FM can be replaced by processed carinata and /or camelina meals in fish diets without affecting fish performance?
5. How much processed carinata and /or camelina meals can be included in fish diets without affecting fish performance at similar FM and /or animal protein contents?
6. How are amino acids in carinata and/or camelina utilized in fish once absorbed?

The above questions were answered by assessing the performance of carinata and camelina seed meals in experimental feeds investigated in RBT and HSB using the following six objectives:

1. Determine process effects on carinata and camelina seed meals' composition, and palatability and digestibility in Rainbow Trout *Oncorhynchus mykiss* (RBT) and Hybrid Striped Bass (sunshine) Bass *Morone chrysops* ♀ x *M. saxatilis* ♂ (HSB).
2. Determine dietary tolerance of glucosinolates and sinapine from carinata seed meal by HSB.
3. Determine how much FM can be replaced in low (20%) animal protein diets of RBT containing FM as the sole animal protein.
4. Determine the maximum inclusion levels of aerobically converted carinata meal followed by a single wash (ACCM) and double washed carinata meal (WCM) without (AC) in low animal protein (20%) diets of HSB.

5. Determine the effects of replacing FM with ACCM in low (20%) animal protein diets of RBT containing FM as the sole animal protein on trypsin activity, protein and amino acid digestibility and bioavailability.
6. Determine the effects of including up to 30% ACCM and WCM in low (20%) animal protein diets of HSB on trypsin activity, protein and amino acid digestibility and bioavailability.

Significance of the Study

Aquaculture has the potential of bridging the gap between the increasing demand for fish protein and the stagnating or declining supply of fish from the wild. The increasing human population and the increasing demand of a high quality protein has increased the shift from other protein sources to fish in human diets and this has positively impacted the expansion of aquaculture as fish supply from aquaculture is more predictable compared to catches from the wild. However, increase in aquaculture production requires a corresponding increase in fish feed production of which the major source of protein has traditionally been fish meal (FM). With stagnating or declining catches of fish species used for fish feed production and the increased competition for FM with other animal production sectors, the need to reduce FM in fish feeds is required because of its increasing cost and low supply. The sustainable source of proteins in fish feeds may be of plant origin and this has directed most attention in fish nutrition research on plant protein sources in fish feeds.

Plant meals and by-products normally cannot fully replace FM in most carnivorous fish feeds because they are low in protein compared to FM and most of them contain antinutrients that have negative consequences to fish performance. The use of

plant meals would therefore require removing the antinutrients and determining how well the nutrients are utilized by the fish. If components of the plant meals not of use to fish are removed, the protein, essential amino acids, and fatty acids contents of the meals are generally increased, relative to the raw meals. Much fish nutrition attention was initially focused on soybean meal as the best replacer of fish meal in fish feeds but this is being expanded to include other protein sources of plant origin such as other oilseeds because soybean meal has also become expensive due to limited supply and competition with other animal feed production sectors.

This study was part of a large oilseed study aimed at evaluating the use of carinata and camelina seed meals as protein sources in the animal feed industry. Carinata and camelina meals were tested for use in the animal production sector because of the potential increased availability of the two seed meals after oil extraction for biofuel production. However, carinata and camelina seed meals are high in glucosinolates, sinapine and crude fiber beyond what most animals, especially monogastrics can tolerate. Oil extraction and removal of antinutrients from carinata and camelina seeds was done by other studies within the large project and this study assessed how well the protein from carinata and camelina seed meals can be utilized by fish with reference to RBT and HSB.

In the first study, the palatability and digestibility of protein and energy of different processed carinata and camelina meals were evaluated in RBT and HSB. In the second study, tolerance of glucosinolates and sinapine from carinata meal was determined in HSB. Based on meal composition and results of the first two studies, we chose to replace FM with aerobically converted carinata followed with a single wash (ACCM) in low (20%) animal protein RBT diets in the third study. In the fourth study,

the maximum inclusion of ACCM and double washed carinata meal (WCM) was evaluated in HSB diets containing similar but low (20%) animal protein. Fifth and sixth studies were follow up studies after the third and fourth studies that were conducted to determine the physiology behind utilization of ACCM and WCM in RBT and HSB, thus explaining the observed differences in the respective growth trials.

Results of this study will avail fish nutritionist with interest in using carinata and camelina seed meals in fish feeds with the latest information on the nutrition quality of meals from genetically modified carinata and camelina seeds. If the nutritional value of carinata and camelina seed meals can be maximized at a low cost, high replacement levels of FM by carinata and camelina meals may be achieved at a low cost and this will reduce feed costs and thus the overall fish production cost.

Limitations and Assumptions of the Study

True digestibility could not be simply measured and only apparent digestibility was measured since we could not quantify any endogenous nutrients in the feces. We assumed that the marker used in digestibility feeds was 100% inert and was homogenously mixed in the feed and feces. The inert marker was also assumed to move at the same rate as other digesta so that the fecal samples obtained were a true representative of the feces. We assumed that the digestive capabilities of all fish were similar since the fish were similar in size and randomly distributed among tanks. Therefore, treatments were assigned randomly to tanks. Feces were collected by stripping fish, assuming that nutrients were fully digested in the distal feces or at least digested to a degree at which digestibility can be equal or better than the digestibility coefficients obtained by indirect methods where fecal samples are collected from the water column.

There are various types of glucosinolates in carinata although sinigrin is the most abundant (> 80%). The nature and / or the composition of their hydrolysis products depend on conditions such as pH, presence of ferrous irons, and myrosinase-interacting binding proteins. These various conditions of glucosinolate hydrolysis and their effects on the nature of glucosinolate hydrolysis products were not measured. Therefore, the nature of glucosinolate hydrolysis products was assumed similar in all fish.

We did not measure any effects caused by handling and anesthesia at the time of sampling on physiological parameters. Therefore, any physiological changes were attributed to changes in diet composition.

We only measured approximate differences in bioavailability not true bioavailability. This is because we sampled blood from different fish and assumed that the digestive capabilities of the fish were similar for similar sized and aged fish.

Organization of the Study

The overall evaluation of carinata and camelina seed meals as novel protein sources in RBT and HSB was divided into six studies, with each study described in chapters 3, 4, 5, 6, 7 and 8. Results of chapters 3 and 4 provided information that was required in chapters 5 and 6. Chapters 7 and 8 were follow up studies after chapters 5 and 6, respectively.

Various experimental procedures were described in chapter 3) to determine process effects on meal composition, protein and energy ADCs, and palatability of processed carinata and camelina meals in RBT and HSB; in chapter 4 to determine the tolerance of glucosinolates and sinapine in HSB; in chapter 5 to determine how much FM could be replaced with ACCM in low (20%) animal RBT diets; in chapter 6 to determine how much ACCM and WCM would be included in low (20%) animal HSB diets; and in chapters 7

and 8 to determine utilization of protein and amino acids to explain the observed growth in chapters 5 and 6, respectively.

Lastly, a summary of statistically analyzed results of the individual studies were compiled, interpreted, and compared to other similar studies, facilitating the overall evaluation of the potential of carinata and camelina seed meals as novel protein sources in RBT and HSB feeds. Conclusions are presented based on meal performance relative to species-specific reference diets not containing any processed carinata or camelina meals.

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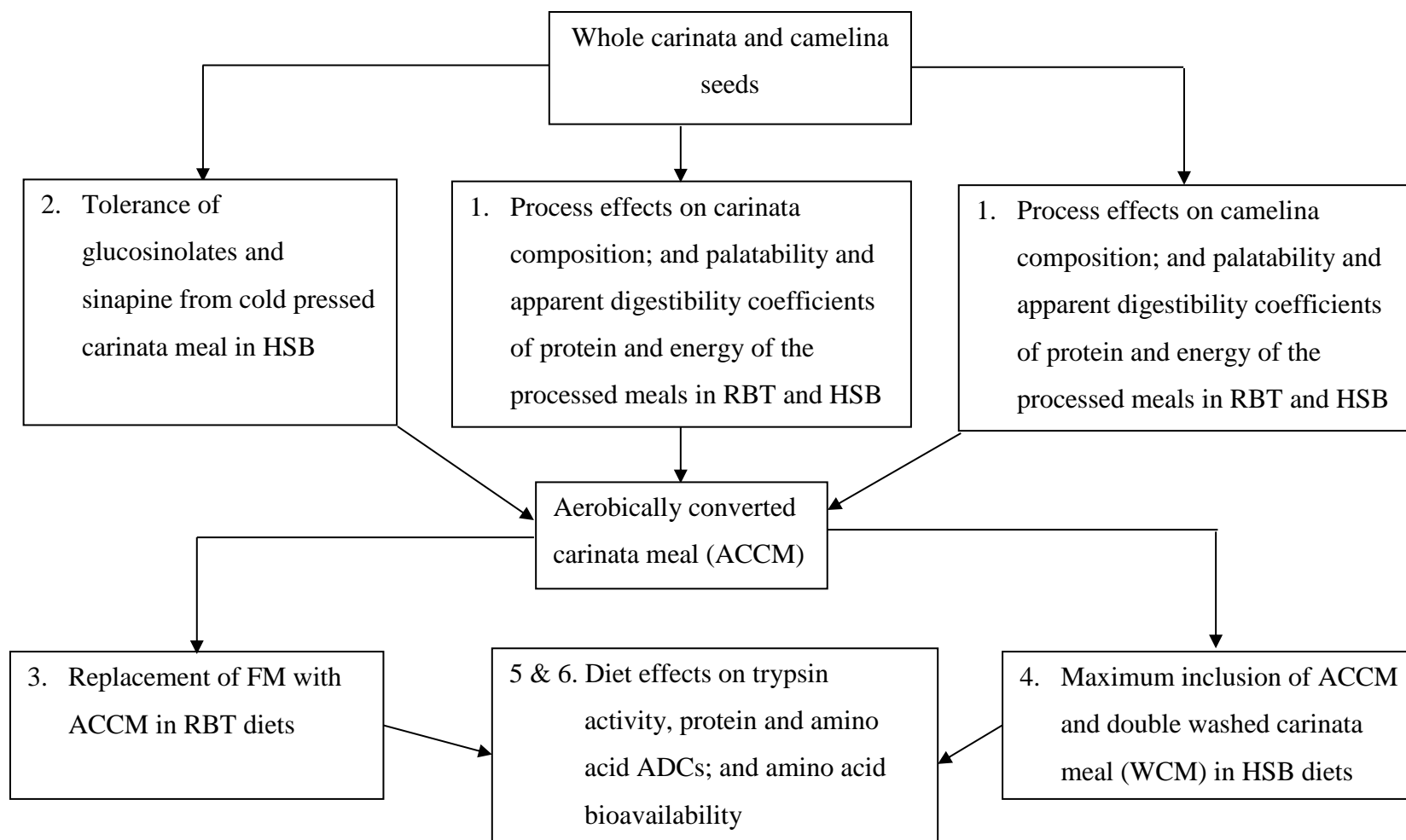
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Figure 1.1. Flow Diagram of the Studies



CHAPTER 2

OVERVIEW OF UTILIZATION AND EFFECTS OF PLANT BASED FEEDSTUFFS IN FISH NUTRITION

Palatability and Digestibility

Ingredient/diet palatability in fish

Fish chemoreceptor cells are organized into taste buds that are found around and in the mouth, pharyngeal cavity, gill arches, and the skin (Whitaker 1971; Gomahr et al. 1992). Only three types of taste buds were previously known to occur in fish (Kinnamon 1987) before the discovery of a fourth type in Cardinalfish *Apogon* sp. in 2004 (Fishelson et al. 2004). Types I and II taste buds are located around the lips and the anterior part of the mouth. Types III and IV are from the middle to the posterior pharyngeal region (Fishelson et al. 2004). Signals from the anterior mouth are transferred to the brain stem by the facial nerve/cranial (CN) nerve VII. The glossopharyngeal (CN IX) and vagus (CN X) nerves transfer signals from the middle and posterior parts of the pharyngeal cavity (Kinnamon 1987). Fish are known to have the basic taste sensations of sweet, sour, bitter and salty. Food items may contain stimulants that evoke feeding behavior such as suction, grasping, snapping, tearing and pinching or suppressants that decrease grasping of food items. Feed stimulants and/ or enhancers increase feed intake and feed deterrents result in feed rejection (Kasumyan and Doving 2003).

A palatable ingredient is considered as one whose taste or flavor is good to be eaten (Glencross et al. 2007). Most feed ingredients of plant origin contain various antinutrients that make them less palatable to fish (Gatlin et al. 2007). Therefore, optimal inclusions of such ingredients in practical diets is always determined to ensure that they

do not reduce diet palatability (Shearer 2000) or nutrient utilization (Gatlin et al. 2007). Some fish species may adapt to diets containing particular anti-palatables (Wybourne and Carter 1999), which shows variation in sensitivity to various anti-palatables among fish. However, loss of production time and reduced feed efficiency during the adaptation period may be a hindrance for use of less palatable diets in commercial fish culture. Therefore, use of less palatable ingredients is unacceptable or they have to be included in diets at low levels that do not affect diet palatability.

Substitution of fish meal and / or ingredients of animal origin in fish diets with plant meals requires processing of plant meals to reduce antinutrients that reduce palatability (Gatlin et al. 2007; Glencross et al. 2007). In addition, feed attractants/stimulants may be added to diets containing unpalatable ingredients to improve feed intake. Compounds that have been studied to improve feed intake in fish include free amino acids, betaine, amines (trimethylamine), nucleotides, nucleosides, sugars, organic acids, alcohols, and aldehydes (Kasumyan and Doving 2003). However, feed stimulants and/ or enhancers may not always yield the same results for all fish species.

Nutrient composition, digestibility and bioavailability for metabolism have limited value to fish if not enough of that ingredient is consumed (Glencross et al. 2007). Non-nutritional factors such as water quality should be maintained at the optimal level for a given species to minimize negative impact on diet consumption (Kasumyan and Doving 2003). Reduced palatability hinders some nutrient assessments of a given feed ingredient. Optimal intake of a given diet is required to determine the optimal digestibility of nutrients of an ingredient/diet. However, if the ingredient makes the diet

less palatable, there are chances of over-estimating the nutrient digestibility of that ingredient under normal physiological conditions, because the enzyme to nutrient ratio increases with less feed consumed, increasing nutrient digestibility.

Determination of palatability in fish

Palatability of feed ingredients/diets in fish has been determined using various direct and indirect methods. Direct methods involve quantifying consumed feed present in the fish stomachs while indirect methods depend on the amount of feed uneaten by fish from a known amount of feed offered, to estimate how much was consumed. Feed intake has been determined by counting intact feed pellets in the stomachs of fish sacrificed shortly after diet consumption and multiplying the number of pellets by a unit pellet weight to determine the amount of feed consumed (Arguello 2011). This method may require fish to be given time to adapt to the diets because an abrupt change in diet may lower feed consumption and may not show whether the fish will adapt to a given diet and how long it will take for the adaption. To avoid sacrificing fish, X-ray radiography has been used to determine the number of intact pellets consumed by fish containing known amounts of compounds such as radio-opaque ballottini glass beads (Kause et al. 2006).

Most studies use quantities of feed consumed at the end of growth trials to determine feed palatability (Deng et al. 2006; De Francesco et al. 2007; Torstensen et al. 2008, Pratoomyot et al. 2010; Sun et al. 2015). These studies may not accurately quantify how much feed is consumed on a daily basis because it may be labor intensive to count unconsumed pellets daily for all feeding times in lengthy feeding trials. Therefore, shorter trials (≤ 3 weeks) aimed entirely at palatability assessment of diets can be done. Uneaten feed from fish tanks is collected and quantified by multiplying the number of pellets with

a unit weight of each dry pellet. Feed consumption is calculated by subtracting uneaten feed from feed offered. The accuracy of this method depends on the ability to collect uneaten feed. It is easier to collect uneaten floating than sinking feed especially with reduced visibility in the water column. Irrespective of the method used in determining diet palatability, uniformity of pellets and their integrity is key to accurately determining the amount of uneaten feed. In addition, fish should be fed beyond satiation during palatability assessments.

Nutrient digestion in fish

Digestion is defined as the process by which nutrients are broken down and solubilized into smaller units that can be absorbed across the intestinal wall to support physiological processes (NRC 2011). Although the basic structural outline of the digestive system of fishes is similar, fish are known to have the most diverse anatomy and organization of cells in the digestive organs (Buddington and Kuz'mina 2000a, b). Broadly, this enables consumption of a variety of food items such as detritus, phytoplankton, zooplankton, micro and macro algae, aquatic plants, insects, crustaceans, mollusks, shellfish, and seeds (Platell and Potter 2001; Lundstedt et al. 2004; De Almeida et al. 2006). However, depending on the nature of the primary ingredients of their feeds, all fish are classified into three categories (NRC 2011): herbivores (plants), omnivores (both plants and animals), and carnivores (animal).

Digestion is carried out both mechanically and enzymatically, with both processes occurring simultaneously along the gastrointestinal tract (GIT). Mechanical digestion is aided by grinding using pharyngeal teeth in some species or by peristalsis and chemical digestion is aided by the various enzymes (NRC 2011). Digestion in fish occurs in the

cavity and along the membranes of the alimentary canal (Ugolev 1985, Ugolev 1989); and within cells lining the GIT (Ugolev and Kuz'mina 1993). Cavital digestion normally occurs in the stomach and intestines (Cyrino et al. 2008). Cavital digestion is carried out by pepsin (pepsinogen) in the stomach and trypsin (trypsinogen), chymotrypsin (chymotrypsinogen), carboxypeptidase A and B, and elastase; lipase and carboxylase α amylase function in the intestine lumen (NRC 2011). Membrane digestion is carried out either by adsorbed enzymes on the intestinal walls such as carboxypeptidases A and B (Srivastava et al. 2003); α amylase (Ribeiro et al. 2002) and lipase (Plotnikov 1984) or by enzymes found on intestinal membranes such as aminopeptidases (Cahu et al. 1998), dipeptidases (Ribeiro et al. 2002), maltase (Ugolev and Kuz'mina 1994), saccharase (Ugolev and Kuz'mina 1994), and alkaline phosphatase (Ribeiro et al. 2002).

Intracellular digestion is carried out by enzymes in the cytosol and lysosomes (Cyrino et al. 2008) of enterocytes such as cathepsin in lysosomes that can fully breakdown protein to amino acids (Nemova 1996) and cytosolic dipeptidases that break down dipeptides into individual amino acids (Ugolev and Kuz'mina 1993).

Digestion in fish is also aided by some symbiotic bacteria in the intestines that can produce enzymes that can help hydrolyze and metabolize proteins, starch, cellulose and non-starch polysaccharides (Kuz'mina 2008; Ray et al. 2009). The dependence of fish on bacterial digestion is small and varies with the composition of food. Herbivore intestines have more bacteria compared to carnivore intestines (NRC 2011). Some fishes such as the Atlantic Cod *Gadus morhua* have digestive compartments that are "closed" with sphincters and are reported to have more bacteria compared to fish with uninterrupted intestines such as Trout *Oncorhynchus* species (Seppola et al. 2005).

Fish intestine length varies depending on the nature of the species food habits. The herbivores have the longest intestines followed by the omnivores and the carnivores have shortest intestines (NRC 2011). The distribution of the various enzymes and their activities in fish also varies depending on the nature of the food consumed (Kuz'mina and Smirnova 1992; Sabapathy and Teo 1993).

The end products of digestion such as dipeptides, amino acids, short chain fatty acids or free fatty acids, disaccharides or glucose, vitamins and minerals are absorbed by diffusion or facilitated transport down a concentration gradient or by active and energy dependent transport against a concentration gradient (NRC 2011). Facilitated and active transport occurs across specialized transporters (Palacin et al. 1998) for that nutrient or a similar group of nutrients (Christensen et al. 1990). After nutrient absorption, the digestion of a particular nutrient is complete and the digestibility of that nutrient from a given feedstuff or diet can be evaluated.

Nutrient digestibility

Digestibility refers to the percentage of nutrients in ingested feed that is broken down into its constituents and absorbed by the fish (De Silva and Anderson 1995). Apparent digestibility is the percentage of nutrients disappearing from the gut without subtracting contributions by microbial fermentation products, endogenous sources and mucous secretions. True digestibility is the percentage of nutrients disappearing from the gut after subtracting nutrients contributed by microbial fermentation products, endogenous sources, and mucous secretions (Ogino and Chen 1973).

Nutrient digestibility in fish can be affected by factors such fish age or size (Usmani and Jafri 2002), water temperature (Azevado et al. 1998; Hua and Bureau 2009),

fecal collection method and type of inert marker (Vandenberg and De La Noue 2001), feed composition (Fountoulaki et al. 2005, Gaylord et al. 2008), and feed processing (Cheng and Hardy 2003). Although different feedstuffs differ in the rate at which they are digested by fish (Fountoulaki et al. 2005, Gaylord et al. 2008), on average, plant meals are digested less and result in the overall reduction of the apparent digestibility coefficients of fish feeds containing plant meals due to the various antinutritional factors (Francis et al. 2001).

Fish meal (FM) is used in fish feeds not only because it is high in protein, lipid and their corresponding building units; and minerals, but also because its nutrients are highly digestible by fish (Miles and Chapman 2006). Most feeds currently formulated for carnivorous fish tend to eliminate, as much FM as possible but fully substituting FM by plant proteins in feeds may not be possible for many carnivorous fishes. Plant meals are not only low in protein compared to FM but they also contain a lot of carbohydrate energy that is not readily used by carnivorous fish and a variety of antinutrients that lower nutrient digestibility (Francis et al. 2001). Fully replacing FM may require highly processed ingredients such as soybean protein concentrate, canola protein concentrate, and land animal proteins and / or supplementing amino acids that are deficient in plant meals (NRC 2011). Processing most plant meals to improve their utilization in fish feeds requires removing antinutrients and elevating proteins by removing components of plant meals such as non-starch polysaccharides (NSPs) like fiber that are high in plant meals and are of very little or no dietary value to most fish.

Antinutritional factors in plant meals with reference to carinata and camelina seed meals and how they affect nutrient digestibility in fish

All plant seeds contain oligosaccharides and NSPs. Carinata and camelina seeds contain other antinutrients such as glucosinolates, phytates (Matthaus 1997; Rakow and Getinet 1998; Pedroche et al. 2004), tannins (Matthaus 1997; Francis et al. 2001), erucic acid (Zubr and Mathaus 2002; Warwick et al. 2006), trypsin inhibitors (Budin et al. 1995; Bell and Rakow 1996) and thiaminase (Tacon 1987). Nutrient digestibility in fish is affected through enzyme inhibition, where the enzymes are prevented from reaching their target nutrients directly by protease inhibitors such as trypsin inhibitors (Krogdahl et al. 1994) or indirectly by NSPs like crude fiber (Ahmad et al. 2004) or some insoluble oligosaccharides. Phytic acids bind proteins at a low pH and cations especially the multivalent cations (Hidvegi and Lasztity 2002) at high pH. Glucosinolates are reported to interfere with mineral utilization (Francis et al. 2001). Thiaminase renders thiamine malfunction by breaking thiamine into constituent molecules (NRC 2011). Erucic acid is not known to affect nutrient digestibility but was reported to be responsible for lipidosis in the epicardial connective tissue of rats (Hendricks 2002).

The primary antinutritional factors (ANFs) in carinata and camelina seed meals can be reduced by ingredient processing or during feed extrusion. Thiaminase and trypsin inhibitors are heat labile and can be greatly reduced by elevating temperatures during extrusion. Romarheim et al. (2005) achieved 76% reduction of trypsin inhibitors in salmon feeds containing 25% soybean meal and extruded at 116-122⁰ C. Singh et al. (2000) achieved complete destruction of trypsin inhibitor activity in a blend of 80% broken rice and 20% wheat bran extruded at 93-97⁰ C. The percentage inactivation of

trypsin inhibitor activity reduced with increasing (>20%) wheat bran in the blend and 60% inactivation of trypsin inhibitors was achieved in 100% wheat bran. Normally, temperatures that can destroy trypsin inhibitors are adequate to destroy thiaminase (Francis et al. 2001). Phytates and tannins can be reduced by dehulling seeds (Griffiths 1991; Francis et al. 2001), fermentation (Mukhopadhyay and Ray 1999), and heating (Hossain and Jauncey 1990; Griffiths 1991). Oligosaccharides are free sugars in plants and include sucrose, raffinose, stachyose, and verbascose (Choct et al. 2010) with monomers ranging from 3 to 10 monosaccharides of mainly glucose, galactose or fructose (Mehra and Kelly 2006). Some oligosaccharides are soluble and are easily digested but generally oligosaccharides such as raffinose, or its monomer galactose, are not utilized by fish (Stone et al. 2003). Because of the low oligosaccharide concentration in most plant meals, the major antinutrients that would affect nutrient digestibility are NSPs due to the high total dietary fiber (NRC 2011). Additionally, the levels of the insoluble NSPs are not changed that much with most ingredient processing methods and may require enzymatic reduction by microbial fermentation or exogenous enzymes.

Nonstarch polysaccharides are a complex group of carbohydrates composed mainly of linked monomers of hexoses and pentoses such as galactose, glucose, arabinose, xylose and mannose joined by α or β -glycosidic bonds (Van Barneveld 1999; NRC 2011). The NSPs are structural components of cell walls and include cellulose, β -glucans, hemicelluloses, pectins, and gums. NSPs are classified into soluble (pectins, gums and some hemicelluloses) and insoluble (cellulose and most hemicelluloses) polysaccharides based on solubility in water (NRC 2011). Based on the method of extraction, NSPs are also classified into crude fiber which is a sum of cellulose, insoluble

hemicelluloses and lignin; neutral detergent fiber (NDF) which is a sum of cellulose, lignin and neutral detergent insoluble hemicelluloses; and acid detergent fiber (ADF) which is a sum of lignin, cellulose and acid insoluble hemicelluloses (NRC 2011; Sinha et al. 2011).

Soybean seeds contain 20-30% NSPs (Macrae et al. 1993), wheat grains contain 25% NSPs and lupin seeds can contain up to 50% NSPs (Van Barneveld 1999). The NSPs are introduced in fish feeds when plant meals are used as fish feed ingredients, their levels in the feeds depend on how much, and the type of plant meals used in the feeds. However, fish and other monogastrics do not produce NSP degrading enzymes (Kuz'mina 1996) and the contribution of symbiotic bacteria may be negligible because most monogastrics do not harbor many microbes. Ruminants harbor more abundant microbes and depend on such symbiotic bacteria for NSP digestion (Van Soest 1994). Cellulose is a structural component of plant cell walls and it can be as high as 33% in plant materials depending on the part of the plant (Sinha et al. 2011). Most monogastrics such as fish have no or very limited capacity to digest cellulose because they do not produce cellulase (Sinha et al. 2011) which microbes such as bacteria and fungi produce (Xiao and Xu 2002). The composition of other NSPs is normally lower than that of cellulose in plants and they include non-cellulosic polymers such as arabinoxylans, mixed linked β -glucans, and mannans, and peptic polysaccharides such as arabinans, galactans, and arabinogalactans (Sinha et al. 2011). NSPs affect digestibility by blocking enzymes from interacting with their target nutrients (Choct et al. 1996), reduce the absorption of sodium ions (Amirkolaie et al. 2005). The insoluble NSPs increase the residence time of digesta (Rainbird and Low 1986; Leenhouwers et al. 2007a, b). Soluble NSPs can

increase rate of passage of stomach contents and delay the intestinal absorption of glucose (Potkins et al. 1991; Shimeno et al. 1992; Kaushik et al. 1995); Refstie et al. 1999; Bach-Knudsen 2001; Hossain et al. 2001; Leenhouwers et al. 2007a, b).

Considering the above effects of NSPs on nutrient utilization in fish and increasing FM costs, research on cost effective removal methods of NSPs in plant meals is necessary to improve the utilization of plant ingredients in fish feeds.

Several NSP degrading enzymes have been utilized in monogastric diets. Choct et al. (1995) supplemented a commercial glycanase product to wheat fed to broiler chickens and observed an improvement in nutrient utilization. Yin et al. (2000) reported improved crude protein digestibility with addition of xylanase to wheat or its by-products in growing pigs. Several studies (reviewed by Castillo and Gatlin 2015) have reported improved nutrient digestibility, feed efficiency and growth when using exogenous carbohydrases in different fish species. Utilization of NSPs by monogastrics using NSP degrading enzymes has been suggested to involve disrupting plant cell walls thus allowing enzymes and water to penetrate cells; reducing of digesta viscosity and stimulation of symbiotic bacterial populations (Sinha et al. 2011). If enzymes are used as ingredients in feed manufacturing, they would be destroyed by heat during feed cook extrusion, or would undergo proteolysis in the stomachs. The best option is to process feed ingredients used in feed manufacture by using exogenous enzymes on the ingredients directly and providing the optimum conditions for them to hydrolyze the NSPs. Another option is to use microbes like bacteria and fungi that can produce NSP degrading enzymes (Xiao and Xu 2002).

Using microbes that degrade NSPs requires identifying, isolating and propagating the appropriate microbes to produce the required NSP degrading enzymes. A method commonly used to produce enzymes is fermentation. Fermentation is where the microbes are mixed with given substrates and the common optimum conditions provided for them to convert the substrates into targeted or usable products such as ethanol, cheese, monosaccharides, etc. The optimum conditions for the microbes to be maintained are temperature, pH, carbon, nitrogen, and carbon dioxide. In addition, fermentation duration should be optimized for maximum conversion of substrates into the desired products. Fermentation is divided into two phases: submerged and solid-state fermentation. Submerged fermentation is the propagation of microorganisms in a liquid nutrient broth. In solid-state fermentation, the microbes are propagated on a high solid substrate (Renge et al. 2012).

Removing NSPs from feed ingredients improves the protein content because NSPs compose a large proportion of plant meals and the microbes themselves are a source of nutrients that improves the essential amino and fatty acid profiles of the meals. Therefore, removal of NSPs improves the utilization of plant ingredients in fish feeds. However, production of high quality plant proteins for fish feed by fermentation is still in its infancy stages and therefore the adoption and utilization of high quality plant ingredients produced by removing NSPs would require that such ingredients be consistently produced on a large scale at a reasonable price. If such technology is improved and consistent results are obtained, then utilization of plant ingredients as protein sources in fish feeds will increase. In order to offset the costs of plant proteins

produced by fermentation, it is important that the final product be high in protein, highly digestible and the nutrients be available for growth and other biological functions in fish.

Generally, tolerance limits in fish feed for the antinutrients in carinata and camelina are less than 5g/kg of feed for trypsin inhibitors and phytates (Francis et al. 2001) and less than 1.4µmoles of GLS/g of feed (Burel et al. 2000a) for Rainbow Trout *Oncorhynchus mykiss* (RBT). The antinutritive effects of tannins and thiaminase in fish are reported, but tolerance limits are not exactly known (Francis et al. 2001). Hendricks (2002) reported lipid accumulation in the epicardial connective tissue in Coho Salmon *Oncorhynchus kisutch* when fish were fed a diet containing 3% of the feed as erucic acid for 6 months or a diet containing 6% of the feed as erucic acid for four months. It is generally recommended not to include levels of NSPs greater than 10 % in fish feed (Dioundick and Stom 1990). However, most commercial carnivorous fish feeds contain ≤ 3% crude fiber. Since plant meals are high in NSPs, reducing them to levels manageable by fish is necessary to increase inclusion levels of plant meals in fish feeds. The suitability of processed plant meal as protein source in fish feeds can be evaluated in many ways. However, determining the palatability and digestibility of feed ingredients is always the first step in evaluating the nutritional quality of an ingredient.

How to determine digestibility in fish

Methods of digestibility determination in fish can be either direct or indirect. Direct methods involve feeding a known amount of feed to a fish and collecting all the fecal matter released in the water column. Since the amount of feed ingested is vital, this approach is designed for determination of digestibility for one fish at a time. Collection of all fecal matter in the water column is difficult because feces may mix with uneaten

feed or disintegrate. The indirect methods use a non-toxic, inert and indigestible marker. The inert marker used in the indirect determination of digestibility should be homogenously mixed in the feed and analysis should be simple and accurate (Austreng et al. 2000). Indirect methods are preferred because there is minimal stress to the fish and numerous fish can be used simultaneously in a single rearing unit, which simplifies replication, and fish are not force-fed (NRC 2011).

Several inert markers have been used in the determination of digestibility in fish and these include metal oxides such as Yttrium oxide (Y_2O_3), chromic oxide (Cr_2O_3), lanthanum oxide (La_2O_3) (Austreng et al. 2000) and titanium dioxide (TiO_2 , Vandenberg and De la Noue 2001); acid insoluble ash such as silica and crude fiber (Morales et al. 1999). A good inert marker should pass through the gut at the same rate as the digesta, should be indigestible and should not affect the metabolism of fish (Austreng et al. 2000). There is no inert marker that can fully meet all the requirements of inert markers to be used in digestibility studies in all fish. Chromic oxide is the most widely used inert marker in fish digestibility studies but has several draw backs (Vandenberg and De la Noue 2001) including: variability in analysis and poor recovery (Riche et al . 1995), differential intestinal transit (Leavitt 1985), and digestive and metabolic effects (Shiau and Chen 1993). Specific inert markers are preferred for use in determining digestibility of certain nutrients such as fatty acids, because they provide more advantages such as ease of recovery and measurement of cholestane (inert marker) together with fatty acids (Sigurgisladdottir et al 1992; Carter et al. 2003). Leaching of the inert marker from the fecal matter should be avoided or minimized because it results in low digestibility coefficients. The ratio of the inert marker in the fecal matter to the inert marker in feed

determines how much feed a unit fecal matter represents (Sugiura 2000). Leaching of the inert marker depends on the integrity of fecal matter as well as how long the fecal matter stays in water before collection.

Several methods are used to collect fecal matter in digestibility studies but are divided into active and passive methods. Active methods are indirect methods of fecal collection where fecal matter or digesta is collected before fish egests it in water and include dissection, stripping and vacuum removal (NRC 2011). Passive methods of fecal collection are direct methods that involve collecting fecal matter from the water column in holding units after fish egestion. Passive methods include screening or filtering tank water, siphoning feces from water (NRC 2011), or one of several other collection methods designed to settle the suspended fecal matter in low-flow apparatus such as the Guelph system (Hajen et al. 1993; NRC 2011).

No fecal collection method is perfect and each method has advantages and disadvantages (NRC 2011). Active methods typically result in under estimation of digestibility because either the fecal matter is not fully digested or the fecal matter is mixed with endogenous material (Hajen et al. 1993, Vandenberg and De la Noue 2001). Passive methods of fecal collection can lead to leaching of nutrients, which results in over estimation of digestibility, or the leaching of the inert marker that results in under estimation of digestibility (NRC 2011). Choice of the method used in fecal collection in fish digestibility studies depends on the available resources and ease of the method, recognizing the potential biases of the method.

Fecal stripping as a method of fecal collection is where a gentle pressure is applied to the abdomen of fish, to expel the distal feces (Glencross et al. 2007). This

method of fecal collection may require knowing the gut transit time of digesta so that feces are collected prior to natural egestion. The gut transit time can be determined by feeding the experimental feeds and monitoring the time at which fecal matter just begins to appear at the anus. Care when stripping fish of feces should be taken to avoid blood or other intestinal fluids mixing with feces or applying too much pressure that may injure the fish. Where egestion of feces in the water column is likely to result in under or over estimation of digestibility due to the inert marker or nutrient leaching, stripping can be used as a more conservative method of fecal collection (Glencross et al. 2007). Stripping is normally conducted until enough fecal material has been obtained for analyses. Adequate fecal material for analysis depends on number of analyses and the methods to be used for each analysis. Fecal samples are normally dried at low temperatures or freeze-dried and then stored in the freezer to avoid nutrient degradation before analysis.

Determining the digestibility of nutrients of a given diet only requires determination of the nutrient and inert marker concentrations in the diet and feces. However, determining the digestibility of nutrients from an ingredient requires incorporation of the ingredient in a reference feed of known digestibility at a given percentage of the total feed and then determining the digestibility of the total feed. This is because ingredients alone cannot be fed to fish during digestibility trials because most of them do not meet all the nutritional requirements of the fish (Kleiber 1961). In addition, the ingredient may not be palatable enough to be consumed as a sole diet or may not have other non-nutritional characteristics required to make it into a stable pellet. The test ingredients are normally included at 30% and the reference diet included at 70% of the

test diet (NRC 2011). Ingredient digestibility can then be calculated using the equations below:

$$ADC_{\text{ref and feed}} = 1 - \frac{\text{Cr}_2\text{O}_3 \text{ in feed}}{\text{Cr}_2\text{O}_3 \text{ in feces}} \times \frac{\text{Nutrient content of feces}}{\text{Nutrient content of feed}}$$

$$ADC_{\text{ingred}} = ADC_{\text{test diet}} + [(ADC_{\text{test diet}} + ADC_{\text{ref diet}}) \times (0.7 \times D_{\text{ref}} / 0.3 \times D_{\text{ingred}})]$$

where D_{ref} is the percentage of the target nutrient or Kcal/g gross energy of the reference diet, and D_{ingred} is the percentage of nutrient or Kcal/g gross energy in the ingredient.

Comparison of digestibility coefficients for feeds and ingredients in a given species can be accurately done if the environmental variables are kept at the optimum levels for that particular species (i.e. recirculating systems). Nutrient digestibility should be between 0 and 100%, with 0% digestibility showing that no nutrients in the feed or a particular ingredient is digested and 100% showing that all nutrients in the feed or ingredient are fully digested.

The ADCs of carinata and camelina seed meals will show how the two seed meals are potentially bioavailable. However, even with high ADCs of the two seed meals, the glucosinolate and sinapine concentrations can limit their inclusion levels in animal feeds due to reduced palatability of the diets.

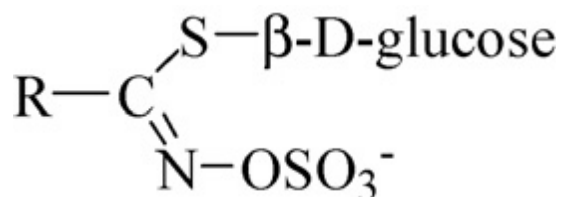
Glucosinolates and Sinapine in Animal Nutrition

Glucosinolates composition of plants

Glucosinolates (GLS) are found in plants of the order Capparales which includes family *Brassicaceae* and are divided into three classes: 1) aliphatic GLS (methionine, isoleucine, leucine or valine), 2) aromatic GLS (phenylalanine or tyrosine) and 3) indole

GLS (tryptophan) based on the type of amino acids precursors that compose the side (R) chains (Redovnikovic et al 2008).

Figure 2.1. General structure of glucosinolates



Source: Tripathi and Mishra (2007)

More than 120 different GLS have been identified (Wittstock and Halkier 2002; Halkier and Gershenzon 2006), however, plants contain only a portion of those GLS in higher concentrations (Redovnikovic et al. 2008). The concentration of GLS in plants greatly differs from one plant species to another and even within different parts of the same plant. The seeds and young leaves tend to have the highest concentrations followed by mature leaves, stems and roots; the lowest concentrations are found in old leaves (Brown et al. 2003). The highest concentration of GLS in the seeds is not due to biosynthesis of GLS in the seeds but it is suggested that GLS are transported from other tissues and deposited in the seeds (Du and Halkier 1998; Chen et al. 2001).

The concentrations of GLS in plants can vary depending on environmental conditions like light (Hasegawa et al. 2000), nutritional status of the plants (Underhill et al. 1980; Kaur et al. 1990), fungal infections, wounding and insect damage (Halkier and Gershenzon 2006). Due to the effect of environmental variability, GLS are assumed to be involved in plant development (Bak et al. 2001; Reintanz et al. 2001; Tantikanjina et al. 2001; Mikkelsen et al. 2004; Tantikanjina et al. 2004). However, the most widely known

function of GLS is to protect plants against herbivores and pathogens (Redovnikovic et al. 2008).

Glucosinolates in carinata and camelina oilseeds and meals

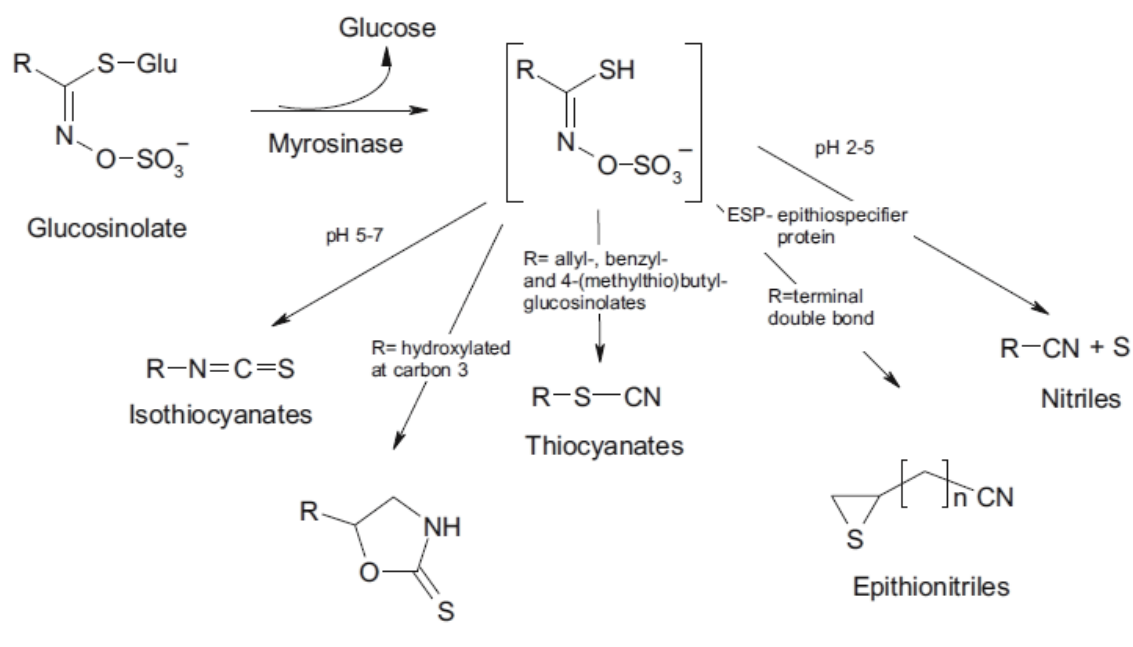
The reported GLS concentrations of carinata and camelina seeds in the 1990s ranged from 87.9-138.7 $\mu\text{moles/g}$ (Warwick et al. 2006) and 13.2-36.2 $\mu\text{moles/g}$ (Lange et al. 1995; Schuster and Friedt 1998), respectively. Plant breeding programs target mainly high erucic acid in both carinata (Warwick et al. 2006) and camelina (Moser 2010) for biofuel production. However, some breeding programs are targeting high erucic acid and high GLS concentrations of carinata seeds where the carinata meal will be used as a bio pesticide (Warwick et al. 2006) and others are targeting high erucic acid and low GLS in the carinata seeds, where the seed meals will be used in animal feeds (Getinet et al. 1997). Carinata seed meal is more unsuitable for use in fish feeds compared to camelina seed meal because carinata seeds contain far more GLS than camelina seeds.

The reported tolerance limit of GLS in RBT is as low as 1.4 $\mu\text{moles of GLS/g}$ of diet (Burel et al. 2000a) and a higher tolerance limit of 11.6 $\mu\text{moles of GLS/g}$ of diet was reported for Turbot *Psetta maxima* (Burel et al. 2000b). However, the concentrations of GLS in carinata (87.6-138.7 $\mu\text{moles of GLS/g}$, Warwick et al. 2006) and camelina (18.5 $\mu\text{moles of GLS/g}$, Russo and Reggiani 2012) whole seeds are high and are concentrated in the meals when the oil is extracted from the seeds. Glucosinolate concentrations in oil seed meals also vary with the method of oil extraction. Solvent extracted meals contain more GLS compared to expeller extracted meals (Tripathi and Mishra 2007). The effective use of carinata and camelina meals in fish feeds requires reduction of GLS concentrations to enable higher inclusions in fish feeds.

Negative effects of glucosinolates in animals

Plants that contain GLS also possess a thioglucosidase enzyme called myrosinase (Lambrix et al. 2001). Glucosinolate and myrosinase are maintained in separate cell compartments and only come in contact when the integrity of the cell is lost due to insect wounding; herbivore and pathogen attack (Grob and Matile 1979), or cell disruption that occurs during grinding of plant meals before feed manufacture (Burel et al 2000a). Combining of GLS and myrosinase produces a thioglucose, sulfate and an unstable intermediate that later rearranges into several degradation products whose composition depends on pH, availability of ferrous irons and the presence of myrosinase-interacting proteins. The final products comprise of isothiocyanates, oxozolidine-2-thiones, nitriles, epithionitriles, and thiocyanates (Redovnikovic et al. 2008).

Figure 2.2. Structures of possible glucosinolate degradation products



Source: Redovnikovic et al. (2008) modified from Wittstock and Halkier (2002)

Glucosinolates themselves are not harmful to animals but its GLS degradation products are harmful (Mawson et al. 1994b; Tripathi and Mishra 2007). Isothiocyanates are bitter (Fenwick et al. 1982; Mithen et al. 2000) and this can reduce feed intake in animals; thiocyanates, thiourea and oxazolidithione interfere with iodine availability to the thyroid, affecting the thyroid function (Wallig et al. 2002). Nitriles are neurotoxic (Balbuena and Llorens 2003). Other reported negative effects of the products of GLS hydrolysis in animals are mutagenicity, hepatotoxicity and nephrotoxicity (Zang et al. 1999; Tanii et al. 2004).

Several studies have reported negative effects of GLS at various concentrations in poultry (Mawson et al. 1994a), rats (Wallig et al. 2002), rabbits (Tripathi et al. 2003), pigs (Corino et al. 1991), humans (McMillan et al 1986), and ruminants (Ahlin et al. 1994). An earlier study by Higgs et al. (1982) reported that low GLS concentrations in RBT feed lowered triiodothyroxine (T_3) and thyroxine (T_4) levels. A later study by Burel et al. (2000a) determined that low GLS concentrations of 1.4 μ moles of GLS/g of RBT diet, reduced plasma T_3 levels and at higher concentrations of between 1.4-19.3 μ moles of GLS/g of diet, growth was affected and the thyroid histology was changed.

Thyroxine is the main hormone secreted by the thyroid gland but it is not bioactive and has to be converted into the bioactive form 3, 5, 3' triiodothyroxine (T_3) by outer ring deiodination (ORD). Outer ring deiodination also converts reverse rT_3 (3, 3', 5' triiodothyroxine) to the bioactive isomer T_3 . Inner ring deiodination (IRD) converts T_4 to rT_3 and degrades T_3 to T_2 . The ORD is considered the activating pathway of T_4 metabolism because it produces T_3 and the IRD is the inactivating pathway because it degrades T_4 and inactivates T_3 (Kuhn et al. 1993). There are three types of deiodinases

that have been characterized in mammals (Leonard and Visser 1986) as well as fish such as RBT (Mol et al. 1998). They include Deiodinase type I, II and III. Deiodinase type I (D1) catalyzes both ORD and IRD and prefers rT_3 as the substrate although it also converts T_4 to T_3 . Deiodinase type II (D2) catalyzes the ORD activities and prefers T_4 as the substrate. Deiodinase type III (D3) catalyzes IRD activities, which are exclusively limited to conversion of T_3 to T_2 (Leonard and Visser 1986).

Thiocyanate anions produced by GLS hydrolysis compete with iodine for active transport across the cell membranes of the thyroid gland to combine with tyrosine residues of thyroglobulin. Vinyloxazolidinethione prevents the binding of two T_2 to form T_4 (Mawson et al. 1994). This results in production of low T_4 in blood and the animals respond by producing more thyroid stimulating hormone (TSH) that increases the follicle activity in the thyroid, resulting in hypertrophy of the thyroid tissue (Mawson et al. 1994b). Rainbow Trout responded to low circulating T_4 and T_3 levels in blood by increasing the activities of D1 and D2, aimed at increasing the T_3 levels. In addition, the activity of D3 was reduced to lower the degradation of T_3 to T_2 . In RBT, D1, D2 and D3 activities were highest in the kidney, liver and brain, respectively (Burel et al. 2000a).

Supplementing feeds containing GLS with iodine increased the T_3 levels (Burel et al. 2000a) in the blood of RBT but not T_4 levels. It is not clear why only T_3 levels were increased and not together with T_4 levels, because T_3 is formed by deiodination of T_4 . Supplementation of feeds containing GLS with T_3 increased plasma T_3 levels (Leatherland et al. 1987; Burel et al. 2000a) more than when iodine was supplemented to the feeds, resulting in improved growth (Burel et al. 2000a).

Due to the negative effects of GLS, some insects have developed compensating mechanisms for GLS ingestion. The Diamondback Moth *Plutella xylostella* produces sulfatase enzymes in its gut that removes the sulfate moiety from the GLS and the resulting compound is inactive and cannot be hydrolyzed by myrosinase. The inactive compound is egested from the moth in feces (Ratzka et al. 2002). The small White Butterfly *Pieris rape* uses nitrile specifying proteins to direct hydrolysis of GLS towards nitriles, which are less toxic than isothiocyanates (Wittstock et al. 2004). Some insects such as the harlequin Cabbage Bug *Murgantia histrionica* can accumulate GLS in their tissues without negative effects to them and use the sequestered chemical as a way of protecting themselves against predators (Allabadi et al. 2002). Insects that use this technique as a defense mechanism normally possess endogenous myrosinase (Jones et al. 2002) or must depend on the myrosinase activity in the guts of their predators.

Glucosinolates are also reported to mediate the immune response of plants of genus *Arabidopsis* in the family *Brassicaceae* against microbial pathogens (Clay et al. 2009). There are no reported effects of pure or semi-purified GLS on the immune responses of fish. However, inclusion of rapeseed meal in Nile Tilapia *Oreochromis niloticus* feeds increased serum alkaline phosphatase activity and reduced superoxide dismutase activity (Zhang et al. 2011). The white blood cell content, hemoglobin and hematocrit increased in Hybrid Tilapia *Oreochromis niloticus* X *Oreochromis aureus* when fed rapeseed meal (Zhou and Yue 2010). Lysozyme activity was reduced in the Japanese Sea Bass *Lateolabrax japonicus* when 20% of FM in their diet was replaced with toasted canola meal (Cheng et al. 2010).

Among higher organisms, ruminants are more tolerant to GLS compared to monogastrics because their gut microflora can transform GLS and or their hydrolysis products (Mandiki et al. 2002). Fish do possess some bacteria in their guts but may not be adequate to detoxify GLS. Negative effects of GLS in diets may depend not only on the concentration of GLS but also on the composition of GLS. Effects of GLS on fish have been studied using rapeseed *Brassica napus* meal (Higgs et al. 1982; Hossain and Jauncey 1989a; Davies et al. 1990; Burel et al. 2000a, b). *Brassica napus* contains mainly (>54%) progoitrin (Schone et al. 2001; Yasumoto et al. 2010, Ban et al. 2017) and no sinigrin (Ban et al. 2017). Other *Brassica* species such as *B. juncea* contains ~71% sinigrin and negligible progoitrin (Merah 2015) and *B. carinata* contains 56.6-95.0% sinigrin (Warwick et al. 2006; Ban et al. 2017) and <2% progoitrin (Ban et al. 2017). Camelina contains 59-70 glucocamelinin (Thacker and Widyaratne 2012; Kosir et al. 2013) and no sinigrin or progoitrin (Thacker and Widyaratne 2012).

Goitrin, the major breakdown product of progoitrin interferes with thyroid metabolism (Mawson et al. 1994b) and allyl isothiocyanates, the major breakdown product from sinigrin are bitter, reducing feed intake (Mithen et al. 2000). Inclusion of 15% camelina in diets of Atlantic Salmon *Salmo salar* reduced diet consumption, resulting in reduced growth after 16 weeks (Ye et al. 2016). Effects of glucocamelinin and other GLS types found in camelina beyond reduction in feed intake are not documented. Dossou et al. (2018) reported a case of extreme inclusion of GLS from rapeseed in fish diets, where fermented rapeseed meal containing 112.8 μ moles of GLS/g of meal was included in diets of juvenile Seabream *Pagrus major* up to 42% of the diet, without reducing feed intake. However, inclusions of 56% of the same rapeseed meal

reduced feed intake, which could be because of the high (74%) FM replacement with fermented rapeseed meal in the diet. On the other hand, few nutrition studies have evaluated the feeding value of carinata (Anderson et al. 2018) and camelina (Hixson et al. 2016a, b; Anderson et al. 2018) meals to fish. In the above studies, inclusion of more than 15% of camelina in fish diets reduced growth. Carinata was tested at one inclusion level of 15% and it did not affect growth and feed utilization after 111 days of feeding. The above diets contained $\geq 25\%$ FM and $\geq 35\%$ total animal meals compared to the study by Dossou et al. (2018), where 42% rapeseed meal containing a high GLS concentration, was tolerated in a diet containing 21% FM as the sole animal protein. Although species-specific tolerance of GLS from rapeseed meal may not be ruled out, the main effect of GLS from camelina and / or carinata is likely reduced feed intake, which may not enable fish to consume enough GLS to disrupt thyroid metabolism.

How to reduce glucosinolates in plant meals

Several mechanisms have been used to lower the GLS content of plant meals. Glucosinolates were reduced by 43.1% in rapeseed meal fermented with fungi *Rhizopus oligosporus* and *Aspergillus* sp. in a solid-state reactor, with the meal to water ratio of 1 to 3, at 25⁰ C under aerobic conditions for 10 days (Vig and Walia 2001). Complete degradation of GLS was achieved when fermentation with *Aspergillus* sp. was carried out at 30⁰ C for 60-96 hours (Rakariyatham and Sakorn 2002). Removal of the β -D glucose could be the mechanism for GLS detoxification by some fungi such as *A. niger* because of its high affinity for β -D glucose (Staiano et al. 2005).

Glucosinolates are also degraded by heating depending on the heating temperature, heating time and preconditioning of the meal such as flaking (Jensen et al.

1995) and HCl addition (Tripathi and Agrawal 1998) to the meal before heating. Wet or pressure cooking is better at degrading GLS than dry heating (Tripathi et al. 2001; Leming et al. 2004). However, drastic heating of rapeseed meal at over 110⁰ C for over 30 min was reported to lower the protein quality of the meal in non-ruminants (Glencross et al. 2004). Therefore, it was suggested that heating should be limited to 30 min at 100⁰ C if the meal is to be used by non-ruminants to maintain the solubility of the protein (Jensen et al. 1995). Extrusion has been reported to reduce GLS (Aumatre et al. 1989; Huang et al. 1995), with wet extrusion degrading GLS better than dry extrusion. Due to variation in the susceptibility of different GLS to heating, the results of lowering GLS in plant meals vary with heating (Jensen et al. 1995; Leming et al. 2004). Glucosinolates can also be reduced by soaking depending on the GLS content, composition (Das et al. 2001; Tyagi 2002) and soaking time (Tyagi 2002). Soaking rapeseed meal with a solution containing copper sulfate was more effective in removing GLS compared to when only water was used (Das and Singhal 2001, 2005) but the role played by copper sulfate was not clear. Soaking is the recommended method for removal of GLS (Tripathi et al. 2000) because it is simple and cost effective.

Sinapine in plants

Sinapine is a storage form of choline and sinapic acid in seeds (Naczek et al. 1998) of plants of the *Brassicaceae* family (Bouchereau et al. 1991). Sinapine is synthesized during seed filling stages and most of it is accumulated in the embryo (Husken et al. 2005). Sinapine synthesis starts by initially converting phenylalanine into sinapate through methylation and hydroxylation steps in the shikimate/phenylpropanoid pathway. Sinapate is then converted by UDP- glucose: sinapate glucosyltransferase

(SGT) into sinapoylglucose. Sinapoylglucose is finally converted to sinapine using sinapoylglucose: choline sinapoyltransferase (SCT, Milkowski and Strack 2010; Jha et al. 2012). Sinapine is hydrolyzed during seed germination to release sinapic acid that is used in production of lignins and flavonoids; and choline used in methylation reactions (Naczek et al. 1998).

Preliminary composition data showed that hexane extracted carinata meal from Agrisoma Biosciences Inc. (Quebec, Canada) contained 4.32, 0.10 and 0.30 mg of sinapine, sinapoylglucose and sinapic acid, respectively per gram of meal. The sinapine, sinapoylglucose and sinapic acid contents in cold pressed carinata meal from Dakota Lakes Research Farm, Pierre, SD were 6.01, 0.18 and 0.45 mg/g of meal, respectively per gram of meal. The sinapine content from Agrisoma and Dakota Lakes were much lower than what was reported (16.6-20.2 mg/g of meal) in carinata meals in the early 1990s (Wang et al. 1998). We did not analyze sinapine, sinapoylglucose and sinapic acid contents in camelina. However, Russo and Reggiani (2012) earlier reported lower sinapine contents (1.58-2.93 mg/g of meal) in 12 camelina genotypes than what they later reported (~1.1-4.8mg/g of meal, Russo and Reggiani 2017) in 47 camelina accessions. The two above studies did not report the sinapoylglucose and sinapic acid contents of camelina. However, sinapoylglucose (Quero et al. 2016) and sinapic acid (Terpinc et al. 2012) have been detected in camelina seeds and cake, respectively. From what we observed in carinata meals, the sinapine contents may always be much higher than the sinapoylglucose and sinapate contents in seeds that contain the three compounds. This could be because sinapine is derived from sinapoylglucose synthesized from sinapic acid.

Negative effects of sinapine and its components in animal feeds

Sinapine and its components are bitter (Ismail et al. 1981) and can reduce diet intake in animals. However, Lee et al. (1984) found sinapine to be less bitter than GLS in rapeseed meal in pigs. Consumption of >1 g of sinapine/kg of diet by brown-shelled egg layers was also reported to cause a fishy flavor in eggs (Butler et al. 1982). Bacteria in the distal gut ferment choline from sinapine into trimethylamine (TMA, March and McMillan 1979). Trimethylamine (TMA), a malodorous compound is then oxidized into an odorless trimethylamine oxide (TMAO). However, a mutation in the flavin-containing monooxygenase 3 (FMO3) gene has rendered brown-shelled egg layers incapable of transforming TMA into TMAO (Honkatukia et al. 2005). This leads to accumulation of TMA in developing eggs (March and McMillan 1979), causing a fishy odor in eggs. Choline from choline chloride was reported not to cause a fishy odor in eggs. However, choline hydrolyzed from sinapine caused a fishy odor because unlike choline from choline chloride that is rapidly absorbed in the small intestines (Goh et al. 1979), choline from sinapine is hydrolyzed by bacteria in the distal intestine (Qiao and Classen 2003), where absorption is very low. This makes choline available for fermentation (Goh et al. 1979). Mutation of FMO3 gene is also responsible for a fishy off-flavor in milk in Swedish red and white dairy cattle (Lunden et al. 2002). Sinapate esters are also reported to undergo oxidation during oil extraction and form complexes with amino acids and proteins in meals, reducing their nutritional value (Naczek et al. 1998).

How to reduce sinapine in seeds/meals

Sinapine can be reduced in seed by genetic modification (Nair et al. 2000; Husken et al. 2005; Bhinu et al. 2009). However, ethical concerns about the use of meals from

genetically modified seeds in animal feeds may be a hindrance. The alternative is to treat plant populations with chemicals that cause mutations and you identify plants with a mutated gene(s) within the sinapine pathway (Harloff et al. 2012). Since sinapine is the last product in the pathway, corresponding reductions in sinapic acid and sinapoyl glucose may not be achieved by random genetic mutation. Sinapic acid and sinapoylglucose are also bitter (Ismail et al. 1981). Therefore, bitterness of the mutated seeds will depend on the presence of sinapic acid and/ or sinapoylglucose and their concentrations in the seeds.

Sinapine can be reduced if meals are fermented with laccase producing fungi (Lomascolo et al. 2012). Sinapine is water soluble (Tan et al. 2011) and a good percentage is likely to be lost in the centrate after liquid fermentation or in the evaporating moisture during drying of the meal (Niu et al. 2015). Solubility of sinapine in water enables inexpensive methods such as soaking/washing to be used to improve the feeding value of sinapine-containing meals. However, the percentage of sinapine and its components removed will depend on the meal to water ratio and the number of soakings/washings used.

Glucosinolates and sinapines being among the most limiting antinutritional factors in carinata and camelina seed meals, their reduction to levels that fish can tolerate can enhance the utilization of carinata and camelina seed meals in fish feeds.

Additionally, if NSPs are also reduced from carinata and camelina seed meals, the two seed meals may substitute more FM in fish feeds on an equal protein basis. Evaluation of the potential of carinata and camelina seed meals as protein sources in fish feeds requires

the two seed meals to be tested in feeding trials against a reference FM-based feed to determine how much FM they can replace, especially in less tolerant carnivorous fishes.

Performance of Rainbow Trout *Oncorhynchus mykiss* and Hybrid Striped (Sunshine) Bass *Morone chrysops* ♀ x *M. saxatilis* ♂ Fed Plant Meals with Reference to Carinata *Brassica carinata* and Camelina *Camelina sativa* Seed Meals

Nutrient requirements of cultured Rainbow Trout and Hybrid Striped Bass

Rainbow Trout are carnivorous and naturally feed on snails, aquatic and terrestrial insect larvae, small forage fish, and crayfish (McCarter 1986). Hybrid Striped Bass (HSB) are also carnivorous and in nature, the young ($\leq 5\text{cm}$) feed on zooplankton such as cladocerans and copepods. In addition to zooplankton, HSB ($\geq 5\text{cm}$) undergo a diet transition and consume some insects until they reach 10cm. After 10cm, HSB start feeding on small fish, mainly Threadfin *Dorosoma pentenese* and Gizzard *D. cepedianum* Shads (Hodson 1989).

Under culture conditions, RBT grower feeds normally contain about 44% crude protein and about 16% lipid (Hardy 2003). The optimal dietary protein content of HSB was determined to be 40% when diets contained 10% lipid (Brown et al. 1992). Both RBT and HSB (NRC 2011) require 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) required by other fish species. Taurine, an amino acid-like (sulfonic acid) compound, is reported to be essential only in the fry stages of RBT (NRC 2011).

The lipid content and composition of RBT and HSB feeds can vary depending on why the lipid is being included in the feed. Because carnivorous fishes depend mainly on

proteins and lipids for nutrient and energy needs (De Silva and Anderson 1994), more lipid can be added to RBT and HSB feeds to meet the energy demands and spare protein for tissue deposition, in which case the protein content of the feeds can be reduced. However, alterations of the lipid content and composition should meet the minimum essential fatty acid requirements of the fish. Carnivorous fish such as RBT and HSB require HUFAs as essential fatty acids in their feeds because they cannot elongate and desaturate C18 fatty acids fast enough into their corresponding HUFAs to meet metabolic requirements due to very low $\Delta 5$ and $\Delta 6$ desaturase activities (Tocher 2003). Rainbow Trout and HSB require 0.4-0.5% and 0.5-1.0% of their diets, respectively, as EPA and/ or DHA (NRC 2011).

The exact quantitative requirements of all the minerals by RBT or HSB are not reported. However, more micro- and macro-mineral requirements have been determined for RBT than HSB (NRC 2011). Generally, fish require 23 minerals in their diets of which seven are required in high amounts (macro-minerals) and 16 are required in small amounts (micro-minerals). Macro-minerals are calcium, chlorine, magnesium, phosphorous, potassium, sodium and sulfur. Micro-minerals are aluminum, arsenic, cobalt, copper, chromium, fluorine, iodine, iron, manganese, molybdenum, nickel, selenium, tin, silicon, vanadium and zinc. Calcium and phosphorous make the majority of fish tissue because they are components of hard tissues of fish like bones and scales (Davis and Gatlin 1996). Absorption of some of the above minerals from water can meet the requirements of fish (Davis and Gatlin 1996). Minerals, whose requirements cannot be met by absorption from water, should be supplemented in feeds. Under culture

conditions, all minerals may be supplemented to diets to ensure adequacy for growth because the supply from water may not be enough or easily be quantified.

Vitamins are micronutrients that are vital for a variety of fish metabolic functions. Vitamins are classified into water soluble vitamins (B and C) and fat soluble vitamins (A, D, E and K) depending on solubility (Halver 2002; NRC 2011). All vitamin requirements of RBT have been determined except vitamins K and B₁₂. The only vitamins/vitamin-like compounds determined in HSB are vitamins A, E, B₂, B₅, C and choline (NRC 2011). Due to lack of data or updated data on the full vitamin composition of all fish feed ingredients, vitamin premixes are normally added to fish feeds to ensure adequacy and to optimize growth (NRC 1993). Another reason is that some vitamins in feed ingredients are degraded by high temperatures involved in feed extrusion (Anderson and Sunderland 2002) or during feed storage (Kubitza and Cyrino 1998). Vitamins such as A, E (Killeit 1994) and C (Anderson and Hedlund 1990) should be incorporated in feeds at higher quantities beyond the required level to compensate for losses due to degradation during feed manufacture and/ or storage. Alternatively, stabilized forms of those vitamins should be used in feeds.

Fish meal as a protein source in fish feeds

Fish meal is high in protein, essential amino acids, minerals, vitamins and digestible energy; and normally meets the nutrient requirements of fish (Miles and Chapman 2006). Not only is FM high in protein, it also has an excellent amino acid profile among fish feed ingredients and does not contain antinutrients (Gatlin et al. 2007). Plant meals are deficient in some essential amino acids such as lysine, methionine and threonine. Therefore, whenever plant meals are used in fish feeds, the deficient amino

acids are normally supplemented, especially lysine, which is always the first limiting amino acid in plant meals (Gatlin et al. 2007).

Solvent extraction (SE) does not remove 100% of the oil from FM and about 6-10% fish oil is reported to be left in SE FM (Miles and Chapman 2006) which can be a good source of HUFAs compared to most plant oils. Vegetable oils mainly contain saturated and monounsaturated fatty acids -- linoleic and linolenic acids -- but no HUFAs (NRC 2011). Therefore, replacement of FM in carnivorous feeds with plant meals requires compensation of lost HUFAs in fish oil lost from FM replacement. Due to the scarcity and high prices of oils high in HUFAs, a mixture of fish oil or another source of HUFAs and vegetable oils such as flax seed oil is used to meet the essential fatty acids and energy needs of fish (Bell et al. 2002).

Fish meal contains high endogenous minerals that are highly digestible by fish compared to minerals in plant meals. Therefore, replacement of FM by plant meals normally requires supplementation of minerals such as phosphorous and calcium (NRC 2011). The low mineral digestibility of plant meals is due to chelating of minerals with antinutrients such as phytic acids (Francis et al. 2001). Even when ingredients that are high in minerals like FM, chicken by-product meal or meat and bone meal are used in fish feeds, mineral supplementation is normally done with mineral premixes to ensure mineral adequacy and to optimize fish growth (NRC 2011).

Due to anticipated differences in the vitamin composition of FM and most plant meals, vitamin premixes should be added to fish feeds irrespective of the ingredients used, with the assumption that the plant ingredients and or FM may not meet all the vitamin requirements of fish (NRC 1993). Addition of vitamins in the diets may help

spare some essential nutrients such as methionine that can be used as a methyl donor in the synthesis of choline if choline is deficient in feeds (Wilson and Poe 1988). Because methionine is limiting in some plant ingredients used in fish feeds (Gatlin et al. 2007), it should be spared for protein synthesis and not required to donate methyl groups in choline synthesis if choline is provided in feeds.

Plant meals as nutrient sources for carnivorous fish feeds

Due to the evolved feeding habits of carnivorous fish, they have higher protein and lipid requirements compared to omnivorous and herbivorous fishes (Wilson 2002). Therefore, prepared feeds for carnivorous fishes are mainly composed of proteins and lipids for nutrient and energy needs (De Silva and Anderson 1994). Typical carnivorous fish feeds are composed of 40-55% crude protein (Wilson 1989; NRC 1993; Wilson 1994) and at least 15% lipid (Wilson 1994), and the feeds normally contain FM and fish oil to supply essential amino acids and essential fatty acids (De Silva and Anderson 1994). The high natural dependence of carnivores on proteins and lipid for energy makes them less efficient at utilizing high levels of dietary carbohydrates in artificial feeds under culture conditions (Wilson 1994; Moon 2001). Therefore, the carbohydrate (starches) content of carnivorous fish feeds normally composes less than 25% of feed (NRC 2011).

Plant meals are lower in protein compared to most animal proteins. In addition, due to essential amino acid deficiencies and low digestibility of minerals in plant meals (NRC 2011), high replacement levels of FM with plant meals in carnivorous fish feeds require essential amino acid and mineral supplementation. Carnivorous and marine fishes also require highly unsaturated fatty acids (HUFA) such as eicopentanoic acid (EPA),

docosahexaenoic acid (DHA) and arachidonic acids (ARA) in their feeds (Tocher 2003) which are deficient in vascular plants (NRC 2011). Although partial replacement of FM in carnivorous feeds with plant meals requires reducing ANFs to levels manageable by fish (Francis et al. 2001) and lowering the carbohydrate energy density of the plant meals (Wilson 1994), maximizing the utilization of plant meals would require removing most of the NSPs which are high in plant meals. The NSPs can compose up to 33% of plant materials (Sinha et al. 2011), and deconstructing NSPs releases nutrients bound in cell walls and also increases the protein and essential amino acid content of plant meals, enabling more FM to be replaced in fish feeds by plant meals on an equal protein basis.

Carinata meal as a protein source in fish feeds

The protein and lipid contents vary in carinata seeds. Pan et al. (2012) obtained protein contents ranging from 25.9 to 30.5% and Xin et al. (2013) obtained an average protein content of 23.8%. Warwick et al. (2006) reported that carinata seed analyzed in 1998 contained an average protein of 34.1%. The lower protein content of carinata seeds after 1998 has been attributed to the breeding work aimed at increasing the oil content of carinata seeds (Xin et al. 2013). The lipid content obtained by Xin et al. (2013) was 40.8%. The essential amino acid profile of the protein in mustard meal (Tacon 1987; Hossain and Jauncey 1989b; Bell and Raynard 1990) is inferior to that of FM (NRC 2011) but similar to other plant proteins. Carinata is high in non-essential amino acid glutamic acid (Malik 1990). The fatty acid composition of carinata seed oil is 30.9-45.7% erucic acid, 2.7-4.2% palmitic acid, 5.1-11.6 oleic acid, 13.7 to 18.9% linoleic acid, 10.2-16.0% linolenic acid, and 6.2-12.0% eicosenoic acid (Warwick et al. 2006). Carinata seed oil does not contain the essential fatty acids (HUFAs) required by carnivorous fish.

Carinata seeds also contain GLS ranging from 87.6-138.7 μ moles/g of whole seed (Warwick et al. 2006) and earlier varieties contained 16.6-20.2 mg of sinapine/g of meal (Wang et al. 1998). Considering the protein contents of carinata reported by Xin et al. (2013) and Pan et al. (2012) after breeding for high oil contents in carinata seed, if all the oil were extracted from carinata, the protein content of the remaining meal would be between 40 and 50% protein. Results of our preliminary analysis of the nutrition composition of carinata meal showed that the protein content of carinata meal ranged from 40.2-46.9%. Glucosinolates ranged from 22-62 μ moles/g of meal and sinapine ranged from 4-6 mg/g of meal. Therefore, depending on how much oil is removed from carinata seeds, the protein content is elevated in carinata meals to levels comparable to rich plant protein sources in fish feeds such as SE soybean meal and cotton seed meal, which contain > 40% crude protein (NRC 2011). In addition, lower GLS and sinapine contents in current carinata varieties may require less processing to be reduced to levels tolerated by fish.

Camelina meal as a protein source in fish feeds

The protein and lipid contents of camelina seeds vary but on average the protein ranges from 26.40 to 29.05% and the lipid ranges from 38.90 to 42.56% (Vollmann et al. 2005). The percentage composition of essential amino acids arginine, phenylalanine, and valine in the protein of camelina seeds (Zubr 2003) is higher than that of FM (menhaden, anchovy and herrings sp.) and the compositions of essential amino acids histidine, isoleucine, leucine, threonine and tryptophan in camelina protein (Zubr 2003) is close to that of FM (NRC 2011). Camelina protein is also high in glutamic acid and aspartic acid (Zubr 2003). Camelina oil is mainly composed of 37.8% linolenic acid, 15.4% gondoic

acid, 14.8% linoleic acid and 5.3% palmitic acid. The erucic acid content of camelina oil is only 2.76% (Zubr 2003). If the average oil content of camelina seeds is 40.73% (Vollmann et al. 2005), removal of most oil from camelina seeds (<1% residual oil) can elevate the protein content of camelina meal to 44.5-49% crude protein. The results of our preliminary analysis of the nutrition composition of camelina meal showed that the protein content of camelina meal ranged from 38.78 to 44.39 %. More oil was left in camelina seed meal than carinata seed meal even when cold pressing and solvent extraction were both used. The average GLS content of camelina seeds reported by (Russo and Reggiani 2012) was 18.5 μ moles/g of whole seed. The higher GLS content in the camelina meal (24-28 μ moles/g of meal) than what Russo and Reggiani (2012) reported could be because the meal we analyzed was de-oiled.

Carinata and camelina seed meals typically contain similar protein contents but because the amino acid profiles do not fully match that of FM, the use of high amounts of carinata or camelina seed meals in fish feeds will require upgrading the protein and amino acid contents by microbial fermentation or amino acid supplementation, especially for carinata seed meal. Camelina seed meal also has other better nutritional properties compared to carinata seed meal because it contains lower GLS. Camelina seed oil contains more linolenic acid than carinata seed oil, which makes it to be of better nutritional value to especially herbivorous and omnivorous fish that can convert linolenic acid into EPA and DHA. Camelina oil is also low in erucic acid that has been reported to be cardio toxic (Slinger 1977).

Feeds containing high erucic acid rapeseed oil caused lipid accumulation and necrosis of the heart muscle in rats (Slinger 1977). However, low erucic acid rapeseed oil

(1-2%) also produced cardiac lesions in rats (Kramer et al. 1975). Because the cardiac lesions were observed even with low erucic acid rapeseed oil, it was not clear if it was erucic acid causing the cardiac lesions. Therefore, it was concluded that the high linolenic acid content of rapeseed oil was responsible for the observed cardiac etiology (Kramer et al. 1992). Lipid accumulation in the epicardial connective tissue in Coho Salmon *Oncorhynchus kisutch* but not necrosis was observed at erucic acid levels of 3% and 6% of feed (Hendricks 2002). However, the fatty acids that accumulated in the epicardial tissue were not reported. De-oiled (<1%) carinata and camelina seed meals may not cause lipid accumulation in the epicardial tissue of fish because the remaining oil in the two seed meals is not high enough to contribute erucic acid levels in the feeds that have been reported to induce epicardial lipidosis.

Growth performance parameters for evaluating the suitability of carinata and camelina seed meals as alternative protein sources in fish feeds

Performance of a fish feed or ingredient can be assessed by measuring weight gain, nutrient utilization, and tissue composition. Growth is measured as a change in weight or length. It is vital that initial fish size is similar and fish have a similar nutritional and genetic background. Determining feed conversion and protein efficiency ratios, and differences in protein and lipid/fatty acid deposition in tissues of fish before and after nutritional studies are helpful in assessing ingredient/feed utilization (NRC 2011).

Differences in growth between the FM-based control feed and test feeds can be due to differences in the physiological responses of fish to the different feed ingredients. Difference in feed ingredients may alter the immune response and activities of digestive

enzymes. Reduction in activities of proteolytic enzymes can be due to ANFs that may directly bind digestive enzymes (as earlier explained), reducing protein and amino acid digestibility. However, other ANFs in plant meals such as fiber prevent access of proteolytic enzymes to target nutrients, which also reduces protein and amino acid digestibility. Reduction in protein and amino acid digestibility may alter the availability of amino acids for metabolism.

Nonspecific Immune System of Fish

Types of immune systems in vertebrates

The vertebrate immune system is divided into innate (nonspecific) and adaptive (specific) immune systems, with the innate immune system acting before the adaptive immune system (Fearon and Locksley 1996). The difference between the two systems is in the receptors required to recognize foreign matter (Bendelac and Fearon 2000; Rice et al. 2002). Innate immunity uses genetically pre-determined pattern recognition receptors (PRRs) to recognize biomolecules (carbohydrates, lipids, and proteins) present in the cell walls of microorganisms, a phenomenon referred to as pathogen associated molecular patterns (PAMPs). Adaptive immunity relies on somatically developed receptors that recognize antigenic patterns that the host has previously been exposed to (Peiser and Gordon 2001; Rice et al. 2002).

The innate immune system comprises of physical barriers such as the skin, mucus and scales (Ingram 1980; Shephard 1994; Ellis 2001); macrophages, monocytes, granulocytes, complement system (Secombes and Fletcher, 1992; Magnadottir 2006), antimicrobial proteins such as mucus trypsin, metal ion chelators (transferrin, caeruloplasmin and metallothionein), protease inhibitors such as α 2-macroglobulin, and

α 1-proteinase inhibitor, lectins, and lytic enzymes such as lysozyme, chitinase, non-specific lysins (Alexander and Ingram 1992; Dalmo et al. 1997). The adaptive immune system relies on T (thymus) and B (bone marrow) lymphocytes that have receptors that recognize particular antigens and therefore can mediate cell-specific immunity (Scapigliati et al. 2003)

Nonspecific immune response of fish

The innate immune system is normally the first defense mechanism protecting against invading pathogens (Narnaware et al. 1994), aimed at maintaining a constant internal environment during growth and development (Magnadottir 2010). In addition to trapping pathogens, mucus also contains nonspecific immune components such as lectins, lysozyme, complement proteins, immunoglobulin M (IgM) and antibacterial peptides (Alexander and Ingram 1992; Rombout et al. 1993; Aranishi and Nakane 1997).

The cells involved in nonspecific immune responses are phagocytes such as granulocytes (neutrophils) and monocytes/macrophages, and non-specific cytotoxic cells (Froystad et al. 1998; Evans et al. 2001; Neumann et al. 2001). Fish are also reported to use epithelial and dendritic cells as innate immune components (Press et al. 1994; Dalmo et al. 1996; Ganassin et al. 1996).

Health indices and nonspecific immune parameters that have been studied in fish include hemoglobin (Hb), hematocrit (Hk), mean corpuscular hemoglobin content (MCHC), alternative complement pathway and lysozyme activity. The choice of immune parameters to study in a given fish may depend on the known or anticipated influence of feed ingredients or immune stimulants in the fish species under study.

Hemoglobin (Hb), hematocrit (Hk) and mean corpuscular hemoglobin content (MCHC)

Hemoglobin (Hb) is a respiratory pigment used to transport oxygen in vertebrates and fish are known to have the highest diversity of Hb (Giardina et al. 2004). Fish stay in a wide range of environments and the Hb content is an interface between fish and the environment (Fago et al. 2002). Hemoglobin quantities can vary with environmental changes such as temperature and oxygen. Changes in environmental variables may also require adjustment of the oxygen binding properties of Hb (Landini et al. 2002). However, not all fish contain Hb. Icefishes (Family *Channichthyidae*) have completely lost Hb and oxygen is carried in physical solution (Sidell and O'Brien 2006). They have compensated for lack of Hb by having high blood volumes and a large diameter of capillaries (Fitch et al. 1984). These features of Hb-less fish enable large volumes of blood to circulate at a high flow rate at a reduced vascular pressure.

In fish with Hb, variations in the concentrations of Hb could be due to changes in temperature and /or photoperiod as the two factors influence oxygen availability (Tun et al. 1986). Hemoglobin concentrations are also affected by agricultural chemicals such as endosulfan (Jenkins et al. 2003). Iron deficiency (Groff and Zinkl 1999) and chronically poor protein nutrition (Dawson and Bortolotti 1997) could also affect hemoglobin concentrations in red blood cells. Dabrowski et al. (2000) obtained Hb concentrations of 10.9g/dL of blood of RBT fed feeds containing 45% animal protein and 100% substitution of the animal protein with cotton seed meal reduced the Hb concentration to 7.9 g/dL of blood.

Hematocrit (Hk) refers to the percentage of the volume of red blood cells in a given volume of blood and normally represents the oxygen carrying capacity of blood

(Houston 1997). Hematocrit is used an indicator of the health status of fish (Allen 1993). Low Hk is a common occurrence in chronically poor protein nutrition (Dawson and Bortolotti 1997). Hematocrit can rise due to an increase in number or swelling of red blood cells (Weber and Jensen 1988). Optimum Hk values for a particular fish species may be difficult to ascertain because the number of red blood cells may change based on the physiological state of the fish such as exercise (Nielsen et al. 1999) or environmental variables such as oxygen levels (Jewett et al. 1991). Optimal Hk depends on the relative velocities of blood flow in parallel adjacent layers of blood under pressure in the blood vessels (shear rate) and blood volume. Therefore, optimal Hk comparisons within and between species is difficult to determine without determining oxygen transport. Oxygen transport is determined by blood volume and hemoglobin concentrations (Gledhill 1992). Generally, fish with high energy demands that can tolerate low ambient oxygen levels, have increased Hk values (Lowe et al. 2000). Hematocrit values in fish may reduce with infections such as fin rot disease (Ziskowski et al. 2008).

In resting RBT, the reported Hk percentage is between 35-40% (Flos et al. 1988; Benfey and Biron 2000) but elevations to 50% or more than resting Hk values have been reported following acute stress (Caldwell and Hinshaw 1994). Increased Hk values ranging from 39-48% in RBT were reported by Dabrowski et al. (2000) and Kiron et al. (2004). In the above two studies, blood was drawn from the caudal vasculature of anesthetized RBT and the increase in Hk may have been due to stress caused by handling and/ or anesthesia. Stress causes red blood cells to swell and / or to be released from the spleen (Gallaughier et al. 1992). Li and Gatlin (2003) reported no change in Hk (44.6-49.4%) with inclusion of brewers yeast at 1-4% of HSB diets. Acerete et al. (2009) also

obtained an Hk value (45.9%) for control HSB similar to what Li and Gatlin (2003) obtained. However, a challenge with *Photobacterium damsela* subsp. *Piscicida*, reduced Hk to 31.6-39.9%.

The mean corpuscular hemoglobin content (MCHC) of red blood cells refers to the average amount of hemoglobin per red blood cell in a given volume of cells. It is calculated by dividing the hemoglobin (Hb) concentration by hematocrit (Hk).

$$\text{MCHC} = \text{Hb (g/dL)} / \text{Hk (v/v*100)}$$

Factors that affect Hb and/ or Hk may also have an effect on MCHC.

Alternative complement activity

Complement proteins work together with nonspecific and specific immune systems to remove blood and tissue pathogens. Several complement components bind and opsonize bacteria, rendering them susceptible to receptor-mediated phagocytosis by macrophages, which express membrane receptors for complement proteins. Other complement proteins elicit inflammatory responses, interface with components of the adaptive immune system, clear immune complexes from the serum, and/or eliminate apoptotic cells (Owen et al. 2013).

Three pathways activate the complement system: classical, alternative and lectin pathways (Morgan and Harris 1999). Holland and Lambris (2002) described the sequence of events involved in the three complement pathways. The classical pathway is more complex compared to the other two pathways. The alternative pathway directly activates C3 when it interacts with certain activating surfaces such as Zymosan and lipopolysaccharide. The alternative complement pathway is activated by splitting of C3 into C3a and C3b. The formed C3b attaches to hydroxyl or amine groups of

carbohydrates or proteins on a foreign cell surface. Factor B, a protein similar to C2, is attached to C3b attached to the bacteria cell wall, and is activated and split into Bb and Ba by another plasma serine protease, factor D. The resulting C3bBb complex functions as the C3 convertase of the alternative complement pathway and is stabilized by the serum glycoprotein, properdin. The C3 convertase causes an amplification of the alternative complement pathway by splitting more C3 into C3a and C3b and becomes the C5 convertase when an additional molecule of C3b is attached to the complex (C3bBbC3b). Splitting of C5 by C5 convertase initiates the formation of the membrane attack complex (MAC) and cytolysis (Holland and Lambris 2002).

The complement system in fish works at low temperatures and is more potent than that of mammals (Sunyer and Tort 1995; Sunyer and Lambris 1998). Studies have shown that complement is required for antibodies to counteract certain rhabdoviruses such as viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus in fish (LaPatra 1996; Lorenzen and LaPatra 1999; Lorenzen et al. 1999). It has also been proven that fish complement can inhibit fungal germination and growth. Fish serum from fish resistant to epizootic ulcerative syndrome can inhibit the germination and/ or growth of the pathogenic oomycete *Aphanomyces invadans*, the primary etiological agent of epizootic ulcerative syndrome (Miles et al. 2001).

The ability of fish alternative complement to lyse a range of red blood cells compared to the human alternative complement that can only lyse rabbit red blood cells (Sunyer and Tort 1995) indicates recognition of a wider range of foreign particles. Glucans (Bagni et al. 2000), n-3 HUFAs (Montero et al. 1998), carotenoids from natural products (Amar et al. 2004) and probiotic bacteria (Nikoskelainen et al. 2003) are among

compounds that are reported to improve complement activity in fish. The above compounds in feed can vary with the type of ingredient used or can be added to feed separately to boost the immune system. Alternative complement can be reduced in fish by low dietary vitamin E (Montero et al. 2001). Panigrahi et al. (2004) observed an increase in alternative complement activity from ~320 µg/ml to ~ 380 µg/ml of serum in RBT due to increase in vitamin E and n-3 HUFAs in diets. Acerete et al. (2009) observed an alternative complement activity of 121.8 U/mL in control HSB. However, a challenge with higher concentrations of *P. damselae* subsp. *Piscicida* increased the alternative complement activity to 140.2-149.5 U/mL.

Lysozyme activity

Lysozyme (muramidase) is an antibacterial enzyme that can lyse mainly gram-positive bacteria but also some gram-negative bacteria (Masschalck and Michiels 2003). Lysozyme is found in bacteriophages, plants, invertebrates and vertebrates; and it is produced by white blood cells (Jolles and Jolles 1984). Lysozyme breaks the β (1→4) glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the cell walls (peptidoglycans) of gram-positive bacteria. The gram-negative bacteria can only be lysed by lysozyme after their outer cell wall have been disrupted by complement and other enzymes, exposing the inner peptidoglycan (Saurabh and Sahoo 2008). Lysozyme is also an opsonin that can initiate the complement system, binding to foreign particles and enhancing phagocytosis (Jolles and Jolles 1984; Grinde 1989). Lysozyme in fish is found in mucus, serum and ova (Ellis 1999).

Fish lysozyme activity can vary with stress. Even within a particular stressful condition, the intensity and duration of stress can determine the amount of lysozyme

activity of a particular fish species (Yildiz 2006). Lysozyme activity also varies with season (Swain et al. 2007), sex (Fletcher et al. 1977) and stage of fish development (Studnicka et al. 1986; Muona and Soivio 1992; Schrock et al. 2001). Lysozyme is also reported to vary with water temperature (Kusuda and Kitadai 1992; Kumari et al. 2006) and salinity (Dominguez et al. 2005; Taylor et al. 2007). Other than providing amino acids to synthesize lysozyme (Kumar et al. 2005; Saurabh and Mohanta 2006), some non-protein nutritional components are reported to indirectly enhance lysozyme activity in fish. Vitamins C (Skjermo et al. 1995) and E (Sahoo and Mukherjee 2002) are known to increase lysozyme activity. Glucans found in plant meals are also reported to increase lysozyme activity in fish (Ai et al. 2007).

Panigrahi et al. (2004) reported lysozyme activity of about 5.9 µg/mL of serum obtained from RBT fed a commercial feed. Panigrahi et al. (2005) later reported an increased lysozyme activity of about 15-17 µg/mL of serum obtained from RBT fed a 50% FM feed. Replacement of 50 and 62.5% of FM with defatted *Jatropha curcas* seed meal in a diet containing 45% protein and 34% FM did not alter lysozyme activity in RBT (Kumar et al. 2011). Jalili et al. (2013) observed an increase in serum lysozyme activity in RBT when 40, 70 and 100% of FM in the control feed was replaced with a blend of plant proteins such as corn gluten, wheat gluten and defatted soybean meal.

Other nonspecific immune responses in fish

Other health indices and humoral nonspecific immune responses of fish include: transferrin, which acts as a bacterial growth inhibitor (Langston et al. 2001), iron chelator (Bayne and Gerwick 2001) and activator of macrophages (Stafford et al. 2001); interferon, which is mainly an antiviral protein (Robertsen et al. 2003); protease

inhibitors, which inhibit proteins secreted by pathogens (Zuo and Woo 1997); anti-proteases, which hydrolyze bacterial protein (Magnadottir et al. 2002); cathepsins, which are proteases (Carnevali et al. 2001). Nonspecific naturally occurring antibodies are also present in fish and act on a range of pathogens (Magnadottir et al. 2002). Reactive oxygen species such as superoxide radicals and hydrogen peroxides from immune cells such as neutrophils and monocytes in conjunction with myeloperoxidase and a halide form the basis of a possible antibacterial system (Secombes 1996). Respiratory burst activity has also been reported in fish as a measure of the release of reactive oxygen species (Lunden et al. 2002). Phagocytes are known to engulf and digest cellular debris and pathogens in tissues and have been observed in fish (Panigrahi et al. 2004).

Modes of action of some nutritional nonspecific immunostimulants in fish

Vitamins A, B₆, C and E; and minerals such as iron, fluoride (Blazer 1992), selenium (Lin and Shiau 2007), and zinc (Lim et al. 2001) are micronutrients that have been observed to enhance disease resistance in fish. Fatty acids (n-3 and n-6) are also reported to affect the immune response of fish (Lim and Webster 2001). The above nutrients also have an impact on the immune response of humans and the modes of action are better studied in humans than in fish.

Vitamin A deficiency is associated with reduced phagocytic and oxidative burst activity of macrophages activated during inflammation (Ramakrishnan et al. 2004), and a low number and activity of natural “killer” cells (Dawson et al. 1999). Vitamin B₆ affects lymphocyte maturation and growth, antibody production and T-cell activity in humans. Lymphocyte mitogenic activity is also affected by vitamin B₆ deficiency (Chandra and Sudhakaran 1990; Rall and Meydani 1993; Trakatellis et al. 1993). Vitamin C helps in

removing reactive oxygen species (ROS). The ROS impair the immune response, lead to loss of cell membrane integrity, change membrane fluidity, and affect cell-cell communication (Hughes 2000). Vitamin C has also been reported to enhance neutrophils and monocytes movements (Anderson et al. 1980) and increase cytokine production and synthesis of immunoglobulin in humans (Jeng et al. 1996). Vitamin E removes free radicals and prevents lipid peroxidation because they are both immunosuppressive. Vitamin E also increases lymphocyte proliferation in response to mitogens and natural killer cytotoxic activity as well as phagocytic activity (Meydani et al. 2005).

Iron is required in regulation of cytokine production and mode of action. Iron is also necessary for myeloperoxidase activity involved in the process of killing bacteria by neutrophils through formation of highly toxic hydroxyl radicals. However, pathogens also require iron and other micronutrients for replication and survival. Therefore, maintaining optimal iron concentrations is required by the host to mount an optimum immune response and to avoid the possibility of excess iron, which may induce free radical-mediated damage and access to iron by pathogens (Oppenheimer 2001). Selenium influences inflammation and virulence formation (Rayman 2000). Selenium was reported to improve antibody titers (Wang et al. 1997) and macrophage chemotaxis (Lim et al. 2001). Zinc increases the chemotaxis of macrophages and non-dietary zinc is reported to increase cellular and humoral parameters (Sanchez-Dardon et al. 1999) such as inflammatory responses (transcriptome profiling) and complement C3-1 protein expression (Hogstrand et al. 2002). Zinc was also reported to improve alkaline phosphatase activity in channel catfish (Scarpa et al. 1992).

Fatty acids are used in production of eicosanoids, which act as messengers in the central nervous system and work as local hormones or signaling molecules to control inflammation and immunity. Eicosanoids include prostaglandins, prostacyclins, thromboxanes, and leukotrienes (Arts and Kohler 2008). Eicosanoids are mainly derived from EPA and ARA and the optimal ratio of EPA: ARA for eicosanoid production is species specific (Sargent et al. 1999). If there is an imbalance of EPA: ARA, increased ARA can stimulate the immune system by producing pro-inflammatory eicosanoids and the reverse is true if EPA is more. The suggested causes for the difference in stimulation are competition of enzymes for fatty acid substrates, different cell type or tissue involved, and the dietary source(s) of fatty acids (Balfry and Higgs 2001).

However, vitamins, minerals and fatty acids are nutrients that do not directly stimulate the immune system but provide substrates and cofactors required for the functioning of the immune system. There are other components of plant meals such as glucans that do not provide nutrients to fish or other advanced animals but are known to directly influence the immune system of animals. Plant meals fermented with fungi species could also stimulate the immune response of fish or other animals because fungi are known to contain β -glucans (Rop et al. 2009). Plant meals fermented with bacteria could stimulate the immune response of animals because of the presence of lipopolysaccharides and peptidoglycans in bacteria cell walls. Lipopolysaccharides and peptidoglycans are highly conserved molecules in unicellular organisms and are not found in multicellular organisms. Therefore, they are regarded as foreign when present in multicellular organisms and will elicit an immune response (Raetz 1990)

Glucans are polysaccharides consisting of glucose as the building blocks and they include glycogen, cellulose and dextran (Duchon 1985). Beta glucans (β -glucans) and β -1-3-D-glucans and / or β -1-4-D-glucans are found in cell walls of higher plants and in seeds of some cereals such as barley and oats. Polysaccharides related to β -glucans and / or β -1-3-D-glucans and β -1-6-D-glucans are also found in lower plants of kingdom fungi such as mushrooms, molds and yeast (Rop et al. 2009). Saccharide receptors in the intestinal walls can distinguish between various saccharides. Beta-glucans are recognized by specific β -glucopyranose receptors present on leukocytes (Duckova et al. 1997). The leucocytes are activated to produce bactericidal compounds such as lysozyme, reactive oxygen radicals and amine oxides. Additionally, the cells produce several cytokines which activate nearby phagocytes and leukocytes required to induce specific immunity (Okamoto et al. 2004). The immunostimulatory activity of glucans increases with increased molecular weight, solubility (Yadomae 2000) and degree of branching (Bohn and BeMiller 1995) and the length of the branch (Saito et al. 1991). The glucans can be part of plant feed ingredients such as barley, oats or added separately as prebiotics or as components of microbes used in fermentation.

Lipopolysaccharides (endotoxins) are found in gram-negative bacteria cell walls and mainly consist of an outer polysaccharide region ("O" antigen), a polysaccharide core region and an inner fatty acid rich region (Lipid A). The polysaccharide region determines the serological effects and the fatty acid region determines the biological function (Raetz 1990; Rietschel and Brade 1992; Erridge et al. 2002). Toll-like receptors (TLRs) do recognize specific conserved regions in bacteria (Bricknell and Dalmo 2005)

and TLR-4 is mainly responsible for causing lipopolysaccharide-induced specific shock in mammals (Chow et al. 1999; Medvedev et al. 2001).

Lipopolysaccharides have also been observed to influence immunity in fish (Bricknell et al. 1997; Robertsen 1999; Erridge et al. 2002), and TLRs have been identified in different fish species (Zhang et al. 2003; Jault et al. 2004; Meijer et al. 2004, Bilodeau and Waldbeiser 2005). Lipopolysaccharides stimulate the proliferation of B-lymphocytes (Salati et al. 1987; Kadoma et al. 1994; Verburg-Van Kemenade et al. 1999), initiate many signal transduction pathways required to produce a variety of inflammatory cytokines in humans and other animals including fish (Secombes and Fletcher 1992; Fujihara et al. 2003). Excess production of cytokines in response to lipopolysaccharides results in septic shock in humans (Callery et al. 1990) but fish are resistant to such shock (Wedemeyer et al. 1968). Lipopolysaccharides stimulate increased lysozyme production (Cramer et al. 2000) and production of antibodies by B-lymphocytes (Elkins et al. 1989).

Peptidoglycans form the inner rigid layer of both gram-negative and gram-positive bacteria and are composed of N-acetylglucosaminyl-N-acetyl-muramic acids cross-linked by peptides. Muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamin) is a conserved subunit of bacterial peptidoglycans and is the one that elicits an immune response in multicellular organisms (Madigan et al. 1997). Peptidoglycans are bound by TLR2/TLR6 heterodimers, peptidoglycan-binding proteins (PGBPs) and Nod (nucleotide-binding oligomerization domain) proteins (Dziarski 2003). Peptidoglycans induce production of chemokines that are proinflammatory mediators (Wang et al. 2000).

Trypsin Activity, Amino Acid Absorption and Bioavailability

Proteolytic enzymes in fish with more emphasis on trypsin

Enzymes are biological catalysts that are proteinaceous and are used to accelerate metabolic reactions in living organisms (Voet et al. 2012). Enzymes that digest proteins are divided into endopeptidases and exopeptidases. Endopeptidases break peptide bonds within the protein molecules without cleaving the terminal amino acids and exopeptidases cleave terminal amino acids (Stevens and Hume 1995). Endopeptidases include pepsin produced by the stomach walls and trypsin, chymotrypsin, elastases I and II, and exopeptidases include carboxypeptidases A and B produced by the pancreas (Kurtovic et al. 2009) and function in the intestine.

Pepsin hydrolyzes bonds adjacent to aromatic amino acids and trypsin hydrolyzes bonds adjacent to arginine or lysine. Chymotrypsin functions like pepsin by hydrolyzing bonds adjacent to aromatic amino acids. Elastase I and elastase II hydrolyze bonds adjacent to aliphatic and neutral amino acids. Carboxypeptidase A hydrolyzes carboxyl terminal amino acids that have aromatic or branched aliphatic side chains and carboxypeptidase B hydrolyzes carboxy terminal amino acids that have basic side chains (Ganong 2009). Pancreatic proteolytic enzymes normally hydrolyze proteins and oligopeptides to peptides of about five amino acids that are further hydrolyzed by aminopeptidases; and later tripeptidases and / or dipeptidases at the membrane brush border and within enterocytes, respectively, into individual amino acids (NRC 2011) before absorption; although sometimes di- and tri-peptides are absorbed (Terova et al. 2009).

Proteolytic enzymes are produced in an inactive form (zymogens) to prevent autolysis of cells producing them and to also act as a timing event in biological activities (Neurath 1989). Pepsinogen and HCl in fish are both produced by oxynticoptic gastric cells and are normally located in the cardiac part of the stomach (Bromgren and Jonsson 1996; Bromgren et al. 1998). Protonation of the active portion of pepsinogen disrupts the electrostatic interactions between the prosegment and the active enzyme, thus allowing the prosegment to undergo conformational changes, which initiate the activation reactions of pepsinogen (Twining et al 1983). Trypsinogen is produced from the exocrine pancreas and is converted initially to trypsin by enteropeptidase (enterokinase) produced by glands in the crypts of Lieberkühn (Zamalodchikova 2010). Enteropeptidase hydrolyzes the peptide bond adjacent to the aspartic acid (Asp)-ASP-ASP-ASP-Lysine sequence in the polypeptide chain covering the active site of trypsinogen (Terpe 2003). However, it was discovered recently that enteropeptidase is also activated by duodenase from proenteropeptidase (Zamalodchikova 2010). Trypsin initially formed from trypsinogen activates the remaining trypsinogen and other proteolytic or non-proteolytic zymogens in pancreatic juice such as chymotrypsinogen, proelastases, procarboxypeptidases, procolipase, and phospholipase A (Halfon and Craik 1998). Pancreatic enzymes work best in alkaline environments created mainly by the HCO_3^- in pancreatic juice (Cooper et al. 2010).

Some fish such as cyprinids lack true stomachs (Barton 2006) but complete digestion of proteins can still be achieved because pepsin and chymotrypsin hydrolyze the same bonds, although complete protein digestion may take longer due to lack of pre-digestion of proteins by pepsin before hydrolysis by pancreatic proteases. Even in fish

with stomachs, more protein digestion occurs in the intestine because there are both endopeptidases and exopeptidases in the intestine responsible for fully hydrolyzing proteins compared to the few bonds hydrolyzed by pepsin in the stomach (Ganong 2009).

Trypsin is the main enzyme in feed utilization and growth (Torrissen and Male. 2000) and occurs in different isoforms in the pyloric caeca and intestine (Torrissen and Torrissen 1985). Different distributions of trypsin isoforms determine genetic-related variation in protein utilization and thus growth performance of different fish species (Torrissen et al. 1999). Differences in isoforms of trypsin caused by differences in temperature have caused different trypsins to work at different optimum temperatures (Torrissen et al. 1998).

Trypsin has one active site in all animals from bacteria to humans, formed by a triad of amino acids: histidine, aspartate and serine (Rypniewski et al. 1994). Trypsin production in fish can be affected by environmental factors such as temperature (Torrissen and Male. 2000), light (Sunde et al. 2004), feed composition (Sunde et al. 2004), and growth hormones (Lemieux et al. 1999). Trypsin production in fish can also be altered genetically by transgenesis (Blier et al. 2002). Because optimal conditions such as light and temperature are provided for maximum growth for a given species and there is no direct control of genetic factors like growth hormones during the fish culture period, differences in the production of trypsin in cultured fish with dietary changes is presumed to be due to differences in the quality of the feeds. Although trypsin is produced in other tissues such as esophagus, gonads, muscle, skin, liver, kidney, trypsin's known central role in fish is protein digestion in the intestine (Lilleeng et al. 2007). Trypsin production in other tissues would be an immune response of fish as it has been

immunohistochemically detected in the intestinal epithelium and several other tissues in fish such as Atlantic salmon *Salmo salar* (Braun et al. 1990).

Increased production of proteolytic enzymes in fish with inclusion of plant meals in fish feeds is a physiological response to the lower nutritional quality of plant meals (Gatlin et al. 2007). The compensatory mechanism of increased proteolytic enzyme production (Santagosa et al 2008) is aimed at maximizing protein utilization from poor quality feedstuffs in order to meet metabolic demands. However, reduced activity of alkaline proteases in the intestines of some carnivorous fish with inclusion of plant meals in their feeds has also been reported (Lilleeng et al. 2007; Santagosa et al. 2008). Trypsin activity is generally higher in fast growing carnivorous fishes compared to slow growing herbivorous fishes (Sunde et al. 2001, 2004) because of the higher protein content of carnivorous fish feeds compared to the protein content of herbivorous fish feeds. However, some studies have reported trypsin activities in herbivorous fish similar or even higher than those of carnivorous fish because of the need for the fish to maximize protein digestive efficiency (Sabapathy and Teo 1993; Hidalgo et al. 1999).

Enzyme activity is defined as the number of moles of substrate converted into products per unit time (NC-IUB 1979). It is a measure of the quantity of active enzymes present at a given time. Enzyme assays measure either the rate at which a given substrate is used up or the rate at which a given product is produced over time (Todd and Gomez 2011). Trypsin activity is normally quantified by measuring the rate at which nitroaniline is formed by cleavage of benzoyl-L-arginine-p-nitroanilide by trypsin (Sajjadi and Carter 2004; Horn et al. 2006; Torrisen et al. 2006; Lilleeng et al. 2007; Khantaphant and Benjakul 2008).

Amino acid absorption

After digestion in vertebrates, nutrients are absorbed by diffusion, facilitated diffusion or active transport (Mailliard et al. 1995). Amino acid and oligopeptide uptake is by facilitated diffusion or active transport using amino acid transporters (Palacin et al. 1998) which are located throughout the fish intestine (Buddington et al. 1997). Similar amino acid transporters are found in mammals and fish but they may differ in their substrate specificities (Collie and Ferraris 1995). Amino acid transport in fish is reportedly lower in marine fish than in freshwater fish due to the increased permeability of their intestinal apical membrane which allows some diffusion of amino acids into enterocytes across the enterocyte brush boarder leading to a significant back-flux of amino acids into the intestinal lumen (Ferraris and Ahearn 1984).

Absorption also depends on the relative concentrations of amino acids in the intestinal lumen (Fearn and Hirst 2006). Crystalline amino acid supplementation in fish feeds normally leads to differences in intestinal amino acid absorption because crystalline amino acids do not need to be digested as they are in a form that can be readily absorbed at rates (Schuhmacher et al. 1993). However, the fast absorption rates of crystalline amino acids leads to inferior growth and poor feed conversions (Cowey and Walton 1988) due to reduced bioavailability of crystalline amino acids caused by the imbalance of amino acids in the general amino acid pool in the blood stream. This leads to reduced anabolism and increased catabolism of the excess amino acids.

Amino acids are transported as a group of similar amino acids using transport systems which are different for groups of similar amino acids (Christensen 1984, 1990). Different kinds of amino acid transport systems have been suggested for acidic (aspartic

acid, glutamic acid), neutral (alanine, asparagine, cysteine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tyrosine, valine and tryptophan), basic (histidine, arginine, lysine), proline (Nassar 1989), and short chain polypeptides (McLean et al. 1999). The initial process of amino acid absorption involves Na-dependent association or binding to carrier molecules (Nassar 1989). Di- and tri-peptides are transported by a single transporter known as PEPTide transporter 1 (PEPT1) and it is reported that the di- and tri-peptides are required to sustain growth and metabolism in fish (Daniel et al. 2006; Verri et al. 2010). The expression of PEPT1 was observed to decrease in fish fasted and increased during refeeding. This has supported the hypothesis that PEPT1 is required to maintain growth (Terovala et al. 2009).

After absorption, amino acids are metabolized according to the metabolic requirements of the fish. Some amino acids can be used to make biomolecules (anabolism) and others will be catabolized for energy. Depending on the relative use of amino acids in a given fish species and life stage, increased amino acid bioavailability will ensure optimum growth.

Amino acid bioavailability

Amino acid bioavailability of a feed or ingredient is defined as the proportion of ingested amino acids that is digested and absorbed in a form that can be utilized for metabolism by the animal (Ammerman et al. 1995). In most cases, digestion of proteins results in the release of free amino acids in hepatic portal vein (Murai et al. 1987) but also small amounts of short chain polypeptides are absorbed and released into the blood stream. Absorption of short chain polypeptides is supported by the use of peptides and polypeptide protein drugs in aquaculture feeds to modulate the immune system (McLean

et al. 1999). Amino acids that enter the amino acid pool or metabolic pool in blood are derived from the digestion and absorption of amino acids from proteins in the feeds or from the degradation of proteinaceous molecules within the body (Cowey and Walton 1989; Kaushik and Seiliez 2010). From the metabolic pool of amino acids, their utilization by an animal normally depends on the metabolic demands of the animal and overall efficiency by which other nutrients are absorbed and combined to meet the metabolic demands of the animal. Amino acid utilization is controlled by the rate at which nutrients are transported across cellular membranes, rate of blood flow, organ uptake and the rates of enzyme activities associated with the different metabolic pathways. These factors are also influenced by endocrine and nervous systems, depending on the needs of the animal (Cowey and Walton 1989).

If the amino acids supplied by the feed are in the right proportion needed by the animal for protein synthesis, most of them will be utilized for anabolic functions. However, if some amino acids in the feed are deficient or not in the right proportion needed for anabolic functions, the amino acids will be catabolized (Weijs 1993) to produce energy and other by-products such as ammonia, carbon dioxide and a bicarbonate. Ammonia is toxic to fish even in small amounts and could be a major factor limiting growth especially in water re-use systems (Francis-Floyd et al. 2010). Amino acid oxidation starts with deamination of the amino acids (Cowey and Walton 1989; Zubay 1993) and the carbon skeleton left is converted into citric acid cycle intermediates or fatty acids and glycogen (Cowey and Walton 1989).

The anabolic functions of amino acids in fish are muscle deposition and formation of other proteinaceous bio-molecules used in the maintenance of the general body

physiological functions (Rodehutscord et al. 1997). Normally, deposition of amino acids into muscle accounts for about 25-55% of the total amino acids ingested (NRC 2011) and it is what is considered the main determinant of the utilization and requirements of amino acids in fish (Cowey and Walton 1989). Amino acids for maintenance of the normal functioning of the body of fish involves the continuous replacement of sloughed intestinal cells, mucins and other secretions; and the production of various metabolites, neurotransmitters, hormones and cofactors (NRC 2011). The requirements of certain amino acids for maintenance out of the total amino acid requirement could be much higher than for protein synthesis when such amino acids are part of bio-molecules that are continuously lost by the fish (Nichols and Bertolo 2008). On average, the amino acids required for maintenance in cultured fishes range from 5-20% of the total amino acids in blood (Rodehutscord et al. 1997; Abboudi et al. 2009; Richard et al. 2010).

After digestion, blood initially passes via hepatic portal vein through the liver, which is the main regulator of amino acid metabolism of higher vertebrates (McDonald et al. 2002). Differences in the amino acids profiles are expected between blood in the hepatic portal vein and blood leaving the liver in the hepatic vein. It is estimated that about 20-50% of amino acids in the blood entering the liver are metabolized in the liver (Hoerr et al. 1993, Matthews et al. 1993).

Differences exist in the rate of absorption of amino acids in the gut and the rate of utilization of the various amino acids in the liver and other tissues. Although crystalline amino acids are absorbed faster than intact amino acids (Cowey and Walton 1988; Schuhmacher et al. 1993), the amino acids in intact proteins are utilized more for anabolic functions compared to the crystalline amino acids (Thebault 1985; Lumbard

1997). The rapid absorption of the crystalline amino acids leads to a peak of amino acids in blood in a short time; faster than the rate at which the body can utilize them and thus, the excess amino acids are deaminated leading to high ammonia production, inferior growths and feed conversion (Yamada et al. 1981, Cowey and Walton 1988). Because inclusion of plant meals in fish feeds lowers or delays protein digestibility due to cell walls forming a physical barrier between the digestive enzymes and the their target nutrients (Ahmad et al. 2004), the overall utilization of amino acids may be lowered.

Most bioavailability studies are done using fish that are large enough to give you enough blood for analysis. The fish are fed a known amount of feed e.g. 1% of their body weight. The feed is finely ground and mixed with water before force-feeding fish. It should be ensured that fish are not stressed before and during force-feeding, because stress reduces the flow of blood to the gut (Thorarensen et al. 1993). Blood is sampled at time intervals such as 0, 3, 6, 9, 12, 24 and 48 h (Espe et al. 1993; Lyndon et al. 1993; Ok et al. 2001; Sunde et al. 2003; Karlsson et al. 2006).

Blood samples for quantifying amino acid bioavailability can be obtained by serial slaughter of a group of similar sized fish (Espe et al. 1993; Lyndon et al. 1993; Ok et al. 2001) or by cannulation of the dorsal aorta (Ok et al. 2001; Sunde et al. 2003; Karlsson et al. 2006) and the hepatic portal vein (Eliason et al. 2007). Dorsal aorta cannulation is performed by placing a cannula through a hole made in the snout and inserting the cannula in the blood vessel at the point where the dorsal aorta begins (Soivio et al. 1975). The hepatic portal vein cannulation involves making an incision into the abdominal cavity to access the gut, inserting a cannula in the hepatic portal vein and then suturing the incision (Eliason et al. 2007). Obtaining blood samples by cannulation is

thought to be the best method of blood sampling as opposed to serial slaughter due to differences in individual physiological capabilities of fish (Carter et al. 2001). However, Ok et al. (2001) did not observe differences in most amino acid concentrations in the dorsal aorta with or without cannulation. Obtaining blood samples by serial slaughter of fish may be better than cannulation where fish are small and where fish are likely not to recover from stress due to surgery if cannulation is not done properly. Serial slaughter requires a large number of relatively uniform sized fish, with the assumption that their digestive capabilities and physiology are relatively similar. Blood is obtained from the dorsal aorta, hepatic portal vein or caudal vasculature at regular time intervals by sacrificing the fish. The fish is initially anesthetized, the abdomen opened and blood is drawn from the hepatic portal vein and the dorsal aorta using heparinized needles fitted on syringes (OK et al. 2001). Blood sampled from the caudal vasculature is a combination of arterial and venous blood from the caudal artery and vein, respectively.

Amino acids in blood enter the interstitial space, from where they are actively pumped into muscle cells (Lyndon et al. 1993). Amino acids required for protein deposition in a given tissue are reflected by the amino acid composition of the tissue synthesized (Kaushik and Seiliez 2010). It is vital that the composition of free amino acid pools in a given tissue is similar to the amino acid composition of the tissue for maximum protein synthesis. Therefore, fish feeds should be formulated to supply the required amino acid concentrations in the right composition.

Some metabolic activities involving amino acids other than muscle synthesis are cell signaling, appetite stimulation, energy utilization, osmoregulation, ammonia detoxification, gut development, antioxidative defense, metamorphosis, neural

development, pigmentation, stress response, reproduction (Li et al. 2009). It is therefore evident that proteins are very vital for maintaining the normal functioning of fish.

However, the requirement of amino acids for maintenance activities is more difficult to determine. To achieve all the functions of amino acids, the required amino acids must be supplied to fish in feeds, should be digested and available for metabolism.

In conclusion, modified carinata and camelina seed meals were evaluated as protein sources in RBT and HSB feeds by initially assessing their palatability, proteins and energy ADCs. Tolerance of GLS and sinapine was then determined. Based on the above studies, aerobically converted carinata meal was selected for use in growth trials. A growth trial in RBT was conducted to determine how much FM could be replaced with ACCM in low (20%) animal protein diets. Another growth trial was conducted to determine how much ACCM and double-washed carinata meal (WCM) would be included in low (20%) animal protein HSB diets. Utilization of nutrients to explain the observed growth responses in RBT and HSB was evaluated by measuring effects of ACCM and WCM on trypsin activity, protein and amino acid ADCs and bioavailability after RBT and HSB growth trials.

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CHAPTER 3

PROCESS EFFECTS ON COMPOSITION OF CARINATA *Brassica carinata* AND CAMELINA *Camelina sativa* SEED MEALS, AND PALATABILITY AND DIGESTIBILITY IN FISHES

Abstract

The nutritional value of carinata *Brassica carinata* and camelina *Camelina sativa* seed meals to fish were assessed by first subjecting cold-pressed carinata and camelina seed meals to extrusion, solvent extraction and aerobic conversion or sequential process combinations to determine which process(es) yielded improved chemical composition. Carinata meals generally yielded more crude protein than camelina meals for a given process treatment. Similarly, the fiber contents of carinata meals were generally lower than those of camelina meals. Largest protein increase in both carinata and camelina meals was due to oil removal and aerobic conversion but not extrusion. Aerobic conversion increased the fiber content of all carinata and camelina meals. However, aerobic conversion reduced glucosinolates of all meals by at least 70% with more reduction observed in camelina meals. Palatability of carinata and camelina meals was improved by solvent extraction. Extrusion improved the palatability of camelina and aerobic conversion improved the palatability of carinata. Protein digestibility was generally higher in carinata than camelina meals for both Rainbow Trout *Oncorhynchus mykiss* and Hybrid Striped (sunshine) Bass *Morone chrysops* ♀ x *M. saxatilis* ♂ but overall protein digestibility was higher in Bass than in Trout. Aerobic conversion reduced the protein digestibility of some carinata and camelina meals but the effect was more pronounced in carinata meals.

Introduction

The ongoing genetic modification of carinata *Brassica carinata* (Taylor et al. 2010) and camelina *Camelina sativa* (Moser 2010) is aimed at increasing oil and erucic acid contents to increase their value for green jet-fuel production. The dry seeds are crushed and oil is extracted, providing meals that can be used in animal feeds. The modification of carinata and camelina will also alter the composition of some antinutritional factors (ANFs). Common to many oilseeds, cereal grains and cruciferous vegetables, carinata and camelina seeds contain glucosinolates (GLS), phytates, (Mathaus 1997; Rakow and Getinet 1998; Pedroche et al. 2004), tannins (Mathaus 1997; Francis et al. 2001) and sinapine (Matthaus and Angelini 2005), in addition to non-starch polysaccharides (NSPs) and oligosaccharides (Saini 1989).

Generally, ANFs in plant meals incorporated in fish diets have been reported to reduce diet digestibility (Francis et al. 2001) and palatability (Krogdahl et al. 2010). Tannins have an astringent bitter flavor; can bind digestive enzymes and complex with nutrients like proteins and minerals (Sandoval and Carmona 1998). Phytic acids bind proteins and cations, especially the multivalent cations (Hidvegi and Lasztity 2002). NSPs such as crude fiber found in plant cell walls, can directly block enzymes, or indirectly slow the rate of digestion (Simon et al. 1996). GLS reduce mineral utilization (Francis et al. 2001). The hydrolyzed products of GLS such as isothiocyanates, are bitter (Fenwick et al. 1982; Mithen et al. 2000) and can reduce feed palatability and intake. Other GLS-hydrolyzed products such as thiocyanates, thiourea, and oxazolidithione affect thyroxine production resulting in reduced growth (Wallig et al. 2002). Sinapine (Naczek et al. 1998) and its components (Ismail et al. 1981) are bitter, which reduce diet palatability.

Tannins and phytates are heat stable and if stored in the outer seed coat, can be reduced by dehulling (Griffiths 1991; Fredlund et al. 1997) or degradation through other processes such as fermentation (Mukhopadhyay and Ray 1999). Sinapine can be reduced by dry or moist heating (Zeb et al. 2006), and soaking in alkaline solutions such as ammonia or calcium hydroxide (Stedman and Hill 1987). Pre-treatments without fermentation can reduce ANFs in plant meals to improve utilization in fish diets, but additional microbial fermentation further deconstructs NSPs to release sugars used by microbes, resulting in protein increase. Plant meals low in ANFs and high in protein can replace more animal proteins on an equal protein basis, especially in less tolerant piscivorous species. NSP and oligosaccharide hydrolysis products such as glucose, galactose, and fructose can also be microbially converted to organic acids such as acetic acid, propionic acid, and butyric acid (Clements et al. 1994) that can be directly used by fish for energy. Overall, ANFs in plant meals must be reduced to increase the utilization of plant meals in fish diets.

The protein contents of carinata (25.9-30.5%, Pan et al. 2012) and camelina (27.1-33.3%, Gugel and Falik 2006) seeds are relatively high, and because of the high oil contents of carinata and camelina seeds, the de-oiled meals are higher in protein. Cold pressing carinata and camelina seeds does not remove as much oil (10-20% residual oil) as do more efficient methods such as solvent (hexane) extraction (<1%), which can be applied as a separate process or following a cold press step. Utilization of de-oiled carinata and camelina meals in fish diets will be limited by their higher fiber (>9%) and glucosinolate contents (13.2-138.7 $\mu\text{mol/g}$, Lange et al. 1995; Schuster and Friedt 1995; Warwick et al. 2006; Russo and Reggiani 2012). The tolerance for glucosinolates is low in most fishes;

reported tolerance limits are in the range of 1.4 to 11.6 μ moles of GLS/g of diet for Rainbow Trout (Burel et al. 2000a) and turbot (Burel et al. 2000c), respectively.

In this study, the Apparent Digestibility Coefficients (ADCs) of protein and energy of diets containing cold pressed, extruded, solvent extracted or aerobically converted and sequential process combinations, for carinata and camelina meals, were determined using Rainbow Trout *Oncorhynchus mykiss* (RBT) and Hybrid Striped (sunshine) Bass *Morone chrysops* ♀ x *M. saxatilis* ♂ (HSB). Palatability of the different meals was tested using RBT. This information will enable the selection of the best processing method(s) for carinata and camelina meals to allow commercial application in fish feeds.

Materials and Methods

Source and preparation of test ingredients. —Carinata and camelina seeds were obtained from Agrisoma Biosciences Inc. (Quebec, Canada) and Willamette Biomass Processors (Rickreall, OR), respectively. The two seed types were processed in the Department of Agricultural Engineering at South Dakota State University (SDSU), where each seed type was cold-pressed (CP) to remove oil at a temperature of 90⁰ C using the M70 oil press (Mondovi, WI) consisting of VFD motor (2HP, 1.5KW) run at 20 Hz and die size of 0.22 inches. Portions of the CP meals were extruded (EX) using a Brabender Plasti-Corder extruder (Model PL2000, South Hackensack, NJ) at 80⁰ C at 50 rpm to enhance the breakdown of fiber components. Portions of the CP meal and the CP and EX meals were then solvent extracted (SE) to ensure that most of the residual oil was removed from the meals. A time–temperature combination of 100⁰ C/90 mins was used for the accelerated solvent extraction process. Portions of the four pretreated meals were then aerobically converted (AC) with fungi (William Gibbons, personal communication,

SDSU) to further increase protein, reduce NSPs, oligosaccharides and other ANFs. The eight test ingredients produced for each meal were CP, CPEX, CPSE, CPEXSE, CPAC, CPEXAC, CPSEAC and CPEXSEAC.

Composition of carinata and camelina meals. —Dry matter (AOAC 2006, method 934.01), crude protein (AOAC 2006, method 972.43), crude lipid (AOAC 2006, method 2003.06), crude fiber (AOAC 2006, method 962.09), and ash (AOAC 2006, method 942.05) contents of carinata and camelina (Table 3.1) meals were analyzed prior to formulating treatment diets. Nitrogen free extracts were calculated as 100- (moisture + crude protein + crude lipid + crude fiber + ash) (NRC 2011). The mineral (Table 3.2) and amino acid profiles (Tables 3.3 & 3.4) were analyzed using AOAC (2006) methods 985.01(a, b, d) and 982.30 (a, b, c), respectively. Gross energy, pH and GLS contents (Table 3.5) of carinata and camelina meals were also analyzed. Gross energy was analyzed with a Parr model 6200 Isoperibol calorimeter (Parr Instrument Company, Moline, Illinois), equipped with an oxygen combustion bomb that combusts 2g of sample. Twenty mL of distilled water, with pH adjusted to 7, was added to 5g of meals, stirred and the pH of the resulting mixture was measured with a YSI Professional Plus pH probe (YSI, Yellow Springs, OH). Glucosinolate contents were analyzed with a Shimadzu (Columbia, MD) HPLC system using a method developed by Betz and Fox (1994) with slight modifications.

Diet formulation and manufacturing. —Reference diets were formulated for RBT or HSB. The RBT reference diet was similar to the #1 reference diet from Barrows et al. 2015 (Table 3.6). The reference diets contained 42% crude protein of which 55% of the diet was composed of fish meal and chromic oxide was added at 0.5% in all diets. Sixteen test diets were formulated by replacing 30% (dry basis) of the reference diet with test

ingredients. All the feed ingredients were ground to particles less than 0.8 mm with a Fitzpatrick comminutor mill (Elmhurst, IL). A single blend of the reference diet without chromic oxide was made in a Leland 100DA70 double action food mixer (Fort Worth, TX) and a weight equivalent to 69.5% of the reference diet (dry basis) was added to the test ingredient (30%, dry basis) with 0.5% chromic oxide and mixed in a Hobart HL200 mixer (Troy, OH). All diets were cold pressed (Hobart 4146 grinder) and the resulting pellets (2.5mm in diameter) were dried with a Despatch UDAF electric conveyor drier (Minneapolis, MN). The diets were stored at -20°C, pending use. Dry matter, protein and gross energy of the diets were analyzed using the aforementioned methods for the meals. Chromic oxide (Cr_2O_3) was analyzed using the method described by Cortes (1979).

Palatability of processed carinata and camelina meals. —Rainbow Trout ranging from 14.1 to 27.4 g, were used to test the palatability of diets containing carinata and camelina meals. Twenty fish were stocked in each in each 106L-tank of a 32-tank recirculating aquaculture system (RAS). Four replicates were used for test diets and eight replicates were used for the reference diet; trials were run twice and four replicates of the reference diet were used for each run. Water quality parameters averaged $18.3 \pm 0.08^\circ \text{C}$, 8.02 ± 0.14 , $7.8 \pm 0.17 \text{ mg/L}$, $0.002 \pm 0.000 \text{ mg/L}$ and $0.23 \pm 0.16 \text{ mg/L}$ for temperature, pH, DO, unionized ammonia and nitrite-nitrogen, respectively. Total tank biomass was measured before and after the trial. Weight gain divided by the number of days of the trial was used to estimate the daily tank biomass. The fish were fed known diet amounts twice per day (AM and PM) in excess of satiation, but consumption was only determined for the morning (AM) feeding. All fish were fed a reference diet for one week to condition them to the system and to estimate baseline tank feeding amounts. The initial pellet check was

done 20 mins following feeding the last tank and tanks having 0 pellets were fed an additional 2g of the diet; tanks were checked again after an additional 20 mins. Residual AM pellets were counted and used to estimate next day's feed amounts for AM feeding. After the first week of fish conditioning, the diets were switched from the reference diet to test diets for 7 days after which the reference diet was again fed for another 7 days before presenting other test diets. Amounts of eaten and uneaten pellets were determined for each tank after the morning feeding. Uneaten pellets were subtracted from pellets fed per tank and used to calculate the average relative diet intake for the seven days as a percentage of the tank biomass to standardize comparisons among diets.

$$\text{Diet consumed} = (\text{pellets fed} - \text{uneaten pellets}) \times \text{average weight of a dry pellet of a particular diet}$$

Digestibility fish culture system and fecal collection. —Ninety RBT ranging from 190-250g, were stocked into each of four 750L tanks and maintained on a flow-through system. Dissolved oxygen, temperature and pH were monitored daily (YSI Professional Plus, Yellow Springs, OH) and individual tanks averaged 10.2 ± 0.8 mg/L, $11.2 \pm 1.1^{\circ}\text{C}$ and 7.6 ± 0.5 , respectively. Unionized ammonia was monitored weekly and averaged 0.001 ± 0.001 mg/L. Fifty HSB ranging from 250-350g, were stocked into each 200-gallon tank and maintained in a recirculating aquaculture system. Dissolved oxygen, temperature and pH were monitored daily (YSI Professional Plus, Yellow Springs, OH), averaging 4.2 ± 0.8 mg/L, $24.4 \pm 1.5^{\circ}\text{C}$ and 7.5 ± 0.3 , respectively. Unionized ammonia and nitrite were monitored weekly and averaged 0.011 ± 0.005 mg/L and 0.11 ± 0.03 mg/L, respectively. Fish were fed an unmarked reference diet for one week before and between exposures to test diets. The test diets were fed for one week after which the fish were stripped to obtain distal feces (Glencross et al. 2007). After fecal stripping, all the fish were again randomly

distributed in the holding tanks and fed the reference diet for one week before presenting the next test diets. All feces stripped from fish in one tank were pooled and the fecal samples were stored at -20°C . After freezing, the samples were freeze-dried using a Labconco Freezone 2.5 freeze dryer (Kansas City, MO) for 72 hours.

Calculation of ADCs. —Dry matter, protein, gross energy, and chromic oxide contents of the dry feces were analyzed using the methods described previously. However, a semi-micro bomb (0.2g of sample) was used for gross energy. Chromic oxide in the ingested diets was assumed to be inert, indigestible, and passage through the digestive tract is the same rate as the dietary nutrients (Austreng et al. 2000). Chromic oxide was also assumed to be homogenously distributed in the diets and the feces. Apparent digestibility coefficients of protein and gross energy were calculated according to the NRC (2011) equations:

$$\text{ADC}_{\text{ref and feed}} = 1 - \frac{\text{Cr}_2\text{O}_3 \text{ in feed} \times \text{Nutrient content of feces}}{\text{Cr}_2\text{O}_3 \text{ in feces} \times \text{Nutrient content of feed}}$$

$$\text{ADC}_{\text{ingred}} = \text{ADC}_{\text{test diet}} + [(\text{ADC}_{\text{test diet}} + \text{ADC}_{\text{ref diet}}) \times (0.7 \times D_{\text{ref}} / 0.3 \times D_{\text{ingred}})]$$

where D_{ref} is the percentage of nutrients or Kcal/g gross energy of the reference diet, and D_{ingred} is the percentage of nutrients or Kcal/g gross energy of the ingredient.

Statistical analysis. —Digestibility data was not replicated due to limited amounts of test ingredients. However, the results were justified by the number of RBT ($n=90/\text{treatment}$) or HSB ($n=50/\text{treatment}$) from which fecal material was collected. The average consumption (percentage body weight) of diets containing carinata and camelina meals was analyzed using repeated measures analysis of variance in Minitab 17. Tukey HSD test was used to identify specific differences among significantly different treatment means. Treatment means were considered significantly different at $P < 0.05$.

Results

Process effects on meal composition. —Processing affected crude protein, crude oil, crude fiber, crude ash and NFE (Table 3.1); mineral composition (Table 3.2); amino acids (Tables 3.3 & 3.4); gross energy, pH and GLS contents (Table 3.5) of carinata and camelina meals. The protein contents of whole seeds of carinata (28.4%) and camelina (28.0%) were similar and increased with oil removal by 59.9 % in CP carinata (45.4%) and by 51.4% in CP camelina (42.4%). Extrusion did not alter the protein contents of CP carinata or camelina meals. Solvent extraction increased the protein contents of CP and CPEX carinata by 6.2% and 8.6%, respectively. Solvent extraction also increased the protein contents of CP and CPEX camelina by 11.1% and 8.1%, respectively. Extrusion of CP meals before SE did not provide additional improvement in protein content.

Aerobic conversion increased the protein content of CP carinata meal by 8.1% but did not improve the protein content of CP camelina meal. The protein contents of CPEXAC carinata meal (49.7%) and camelina meal (49.2%) were similar. The protein content of CPSEAC carinata meal (56.9%) was slightly higher than that of CPSEAC camelina meal (53.1%).

Cold press removed 81.8% of oil from carinata and 79.5% of oil from camelina seeds. Solvent extraction removed almost all the oil in CP and CPEX carinata and camelina meals to less than 1.0% of the oil in whole seeds. Extrusion did not change the oil contents of either CP meal. Aerobic conversion also reduced the oil content of CP carinata meal by 11.5% but unexpectedly increased the oil content of CP camelina meal by 8.5%. Aerobic conversion increased the oil content of CPEX carinata meal by 57.1% but lowered the oil content of CPEX camelina meal by 20.5%. Aerobic conversion increased the oil content

of CPSE carinata meal by 20% and the oil content of CPEXSE carinata meal by 16.7%. Improvements in oil contents by AC in CPSE (0.1-0.7%) and CPEXSE (0.3-0.7%) camelina meals as a percentage of the meals, were insignificant.

Mechanical cold pressing reduced the crude fiber content of carinata seeds by 29.3% but increased the crude fiber content of camelina seeds by 23.7%. Extrusion and SE did not have any effect on the fiber content of the CP carinata and camelina meals. However, AC increased the fiber content of all the base meals.

Cold pressing increased the crude ash content of both carinata and camelina meals but EX and SE did not change the ash content of CP meals. Aerobic conversion reduced the ash contents of both carinata and camelina meals. Nitrogen free extracts (NFE) in both carinata and camelina (Table 3.1) meals were affected by different processing methods. Nitrogen free extracts increased in both carinata and camelina meals with oil removal methods but not EX. Aerobic conversion of carinata and camelina meals reduced the NFEs of each base meal except CPEXSEAC carinata.

The mineral profile showed that oil removal by CP and SE increased the mineral contents of both carinata and camelina meals. Aerobic conversion generally reduced phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), manganese (Mn). There was almost no effect of processing on the sulfur (S) content of the meals but sodium (Na), copper (Cu), and iron (Fe) increased with AC.

The amino acid contents of CP carinata and camelina meals were slightly improved by SE but the largest increase in amino acid contents was by AC. Essential amino acids (i.e. histidine, isoleucine, leucine, lysine, methionine and tryptophan, plus cysteine [conditionally essential], were generally higher in carinata than in camelina meals. The

other essential amino acids (i.e., phenylalanine, threonine, and valine) in carinata meals were similar to camelina meal. There was an improvement in tyrosine with AC in camelina meals. Unlike most plants meals (Li et al. 2011), carinata and camelina meals contained taurine.

Gross energy of both carinata and camelina meals was reduced by oil removal but was unaffected by AC. The pH of AC carinata and camelina meals was more acidic than the pH of the unconverted meals. Glucosinolates were reduced by AC in both carinata and camelina meals, with more reduction ($\geq 97\%$) observed in camelina compared to carinata ($\geq 70\%$) meals. Phytic acids contents of camelina did not change with SE but increased in SE carinata meals. Aerobic conversion lowered phytic acid contents of all carinata base meals. Phytic acid was reduced in CP, CPEX and CPEXSEAC but not CPSE with AC in camelina meals.

Carinata palatability. —The palatability (Figure 1) of diets containing CPEXSE was similar to that of diets containing CPSE, CPEXAC, CPSEAC and the reference diet but higher ($p < 0.001$) than that of diets containing CP, CPEX, CPAC and CPEXSEAC. Palatability of the diet containing CPEXSEAC was similar to that of the reference diet but higher than that of diets containing CP and CPEX. The palatability of the diet containing CPSEAC was similar to that of diets containing CPAC, CPEXAC, CPEXSEAC, CP and the reference diet but higher than that of the diet containing CPEX. The palatability of diets containing CP and CPEX were similar. The general palatability of carinata meals gradually reduced ($p = 0.045$) with days of feeding.

Camelina palatability. —The palatability (Figure 2, $p < 0.001$) of diets containing CPEXSEAC was similar to that of diets containing CPEX, CPSE, CPEXSE, CPSEAC and

the reference diet but higher than that of diets containing CP, CPAC and CPEXAC. Palatability of the diet containing CPEXSE was similar to that of diets containing CPEXAC, CPSEAC, and CP, and the reference diet but higher than that of the diet containing CPAC. The palatability of diets containing CP, CPEXAC, and CPAC were similar. The general palatability of camelina meals gradually reduced ($p=0.043$) with days of feeding.

Meal digestibility in Rainbow Trout. —The ADC of protein in carinata meals (73.9-92.4%) was generally higher than that of camelina meals (65.1-87.0%, Table 3.7). The only camelina meals with ADCs similar to that of processed carinata were CPEXSE and CPSEAC. Generally, EX improved the protein ADC of CP carinata and camelina meals. Solvent extraction only improved the protein ADC of CP carinata. A sequential combination of EX and SE only improved the protein ADC of CP camelina. Aerobic conversion did not improve the protein ADC of any carinata meal. However, AC improved the protein ADC of CP and CPSE camelina meals.

The ADC of gross energy (Table 3.7) was more variable in relation to processing methods in camelina (32.4-64.3%) than in carinata (50.2-66.6%) meals. Extrusion and SE when applied separately increased the ADC of gross energy in CP carinata meals by 16.6%. However, only EX improved the gross energy ADC of CP camelina meal (35.4%) but not SE. Gross energy ADC was improved with AC of CP and CPEXSE meals in both carinata and camelina. However, gross energy ADC was only improved by AC in CPSE camelina but not CPSE carinata. Aerobic conversion did not improve gross energy ADC in any of the CPEX meals.

Meal digestibility in Hybrid Striped Bass. —The protein ADCs of carinata meals (78.6-98.9%, Table 3.8) were also generally higher than for camelina meals (68.4-92.5%) except for CPEXAC camelina that was similar to the carinata counterpart and CPEXSEAC camelina that was higher than the carinata counterpart. Extrusion and SE, when applied separately, only improved the protein ADC of CP carinata meal. A sequential combination of EX and SE only improved the protein ADC of CPSE camelina. Aerobic conversion did not improve the protein ADC of any pre-treated carinata meal. Aerobic conversion improved the protein ADC of all pre-treated camelina meals except the CP meal.

Discussion

A higher protein increase was observed in CP carinata (59.9%) than in CP camelina (51.4%) primarily because 81.8% of the oil content (42.4%) of carinata seeds was removed compared to 79.5% of the oil content (36.6%) of camelina seeds. There was no effect of SE on the protein and oil contents of both carinata and camelina meals. Solvent extraction removed almost all the oil from CP (0.5%) and CPEX (0.6%) carinata meals, and CP (0.1%); and CPEX (0.3%) camelina meals, thereby increasing their protein contents. The protein contents in both CPSE ($\leq 0.5\%$ residual oil) carinata (48.2%) and camelina (47.1%) meals were higher than those of most SE oilseed meals reported in NRC (2011) such as canola meal (38.0), sunflower (32.3%) and linseed meal (35.0%), SE cotton seed meal (41.7%) but comparable to those of SE soybean meal with hulls (44.0%) or toasted and SE soybean meal without hulls (48.5%). A sequential combination of EX and SE did not increase the protein contents of both CPSE carinata and camelina meals, which further showed that EX did not play a role in increasing the protein contents of either meal.

Aerobic conversion increased the protein content of all carinata and camelina meals except for CP camelina and it is not clear why the protein content of CP camelina did not increase with AC. The protein increase in AC carinata and camelina meals could have been due to a wash removal of soluble components of the meals but not due to reduction in fiber as anticipated because the fiber content of all meals increased with AC. A slightly higher protein content of CPSE carinata than that CPSE camelina resulted in a higher protein content of CPSEAC carinata than CPSEAC camelina.

At least 80% of the oil was removed from both carinata and camelina seeds by CP and the need for an additional 18.8-19.7% of oil removal by SE will depend of the cost of the process and the value of the additional oil. Changes in the oil contents of AC carinata and camelina meals were insignificant when expressed as a percentage of the meals. However, the changes in the oil contents with AC were high because of the low oil contents of the unconverted meals and any changes in the AC meals resulted in a high percentage change of the oil contents of the base meals. It is not clear why the oil content of CPEX carinata meal increased with AC.

Cold press reduced the crude fiber content of carinata seed but not camelina seeds. We expected crude fiber to be concentrated with oil removal but the reduction in crude fiber in carinata with oil removal may suggest a difference in the type of fiber between carinata and camelina. The crude fiber contents of all AC carinata and camelina meals increased most likely due to the removal of the soluble components of the meals in the supernatant after centrifugation and very low or no degradation of crude fiber by microbial activity.

The ash contents of unconverted carinata and camelina seeds increased with oil removal that concentrated the remaining nutrients. The ash content in all ACcarinata and camelina meals was reduced due to the liquid AC process that removed the soluble components of the meals. More solubility of NFEs in water than oil led to concentration of NFEs with oil removal and a reduction in NFEs with AC.

Oil removal generally concentrated minerals in both meals. The wash step involved in AC reduced P, K, Ca, Mg, Zn and Mn; suggesting that these minerals were loosely bound. The minimal reduction or an increase in Na, S, Cu, and Fe in both carinata and camelina suggests that these minerals are tightly bound.

The amino acid profiles of whole seed carinata and camelina were concentrated by oil removal. The concentration of most amino acids was further increased by AC due to the wash removal of the soluble seed components. Tryptophan in CPSE, and histidine and tryptophan in CPEXSE were reduced by AC in carinata. Aerobic conversion also reduced arginine, histidine, lysine, methionine and tryptophan in CP, tryptophan in CPSE and arginine and tryptophan in CPEXSE in camelina. Reduction of the above amino acids may not suggest that they are unbound or part of unstructured proteins in the seeds, because the effect of AC would have been similar for all pretreated meals. However, it is worth noting that AC reduced more amino acids in pretreated camelina than carinata meals.

Cold pressed carinata had a better essential amino acid profile than CP camelina and this may suggest that carinata is of better nutritional value than camelina. The amino acid profiles of other processed carinata and camelina meals largely depended on how much more residual oil, soluble and insoluble carbohydrates were removed with processing. Without further processing, the concentrations of arginine, histidine,

methionine, threonine, tryptophan, and cysteine of CPSE carinata (48.2% protein, 0.5% oil) were close or more than those of solvent extracted soybean meal with hulls (SE-SBM). The concentrations of sulfur amino acids (methionine and cysteine) of CP carinata and camelina were higher than those of most plant meals reported by NRC (2011). This could be due to sulfur amino acids acting as a source of sulfur needed for GLS, involved in plant defense mechanism (Halkier and Gershenzon 2006). Sulfur in GLS may represent 1.7 to 8.0% of the total sulfur content of *Brassica* species (Fieldsend and Milford 1994; Blake et al. 1998). For CPSE camelina meal (47.1% protein, 0.1% oil), the concentrations of arginine, methionine, threonine and cysteine were close or more than the concentrations of the same amino acids in SE-SBM.

Carinata and camelina meals contain taurine unlike most plant meals (Li et al. 2011), and this renders them more beneficial for use in diets especially of carnivorous fish. Taurine is synthesized in fish (Yokoyama et al. 1997) and plays a critical role in osmoregulation (Buentello and Gatlin 2002) superseding other functions such as bile salt (taurocholate) synthesis (Huxtable 1992). Taurine requirements in fish increase with increasing salinity (Yokoyama et al. 2001). Carnivorous fish cannot synthesize enough taurine to meet requirements for fast growth under culture conditions or if diets contain high amounts of plant meals (Gaylord et al. 2006). Therefore, plant based diets need to be supplemented with taurine. Because taurine is synthesized from methionine (Yokoyama et al. 2001) via cysteine (Yokoyama et al. 1997), having carinata and camelina meals in diets would reduce the need to supplement methionine and cysteine in the diets.

Gross energy of both carinata and camelina seeds was reduced by oil removal because oil is more energy dense compared to proteins and carbohydrates (Craig and

Helfrinch 2002). The pH of AC meals was lower than that of unconverted meals because AC was carried out at a pH of 3. The low pH of feed ingredients/feed may reduce the amount of hydrochloric acid the animal produces to reach the optimal pH required for protein digestion in the stomach (Eidelsburger 1997; Mroz et al. 2000). However, low pH of feed ingredients may impart a sour taste to the diets, consequently lowering voluntary feed intake as reported for RBT (Fauconneau 1988). Glucosinolates were reduced by AC in carinata and camelina meals because crushing seeds breaks the “compartments” containing myrosinase and GLS (Grob and Matile 1979), which facilitates the hydrolysis of GLS by myrosinase when water is added. The full break down of the compartments enclosing myrosinase and GLS was not likely attained in carinata or the catalytic activities of myrosinase in carinata and camelina are different, resulting in less GLS hydrolyzed in AC carinata than camelina meals. Phytic acid increased in CPSE and CPEXSE carinata because EX further broke the seeds mechanically, that may have been partially broken by grinding, enabling better access to phytic acid by solvents. However, phytic acid is polar due to the negative charges of the phosphate groups attached to inositol (Barrientos and Murthy 1996) and cannot readily dissolve in hexane, which is nonpolar (Richez et al. 2013). This resulted in no reduction but concentration of phytic acid with EX and SE, and a reduction of phytic acid with AC. Extrusion and SE did not increase the phytic acid concentration of any pretreated camelina meal, suggesting a difference in the storage sites of phytic acid in carinata and camelina seeds. The difference in storage sites may have resulted in differences in phytic acid breakdown during processing before AC, which may have resulted in differences in accessibility by water and thus a generally better extraction during AC of phytic acid in carinata than camelina meals.

Palatability of carinata and camelina meals generally improved with SE. Aerobic conversion only improved palatability of meals that were not SE. Higher palatability was generally expected in AC meals than unconverted meals due to degradation of GLS and the wash removal of their breakdown products but it instead improved more with SE that did not significantly lower the GLS contents of the meals. Dai and Lim (2014) suggested that oil extraction by hexane probably affects the GLS-myrosinase system by inactivating myrosinase and or hydrolysis/leaching of sinigrin with oil extraction. However, GLS did not decrease so much with SE in CPSE and CPEXSE meals. Additionally, higher palatability was expected in AC meals because of the anticipated sinapine reduction due to its water solubility (Tan et al. 2011). Palatability was only measured in the morning and based on what we have observed in other palatability studies with RBT of the same size where consumption is measured twice or thrice a day, the morning feeding normally accounts for 30-45% of the daily ration. With a 30-45% consumption of the daily ration in the morning, a daily feed consumption of 4.5-6.2% of the body weights of fish was projected for diets containing carinata and camelina meals in the present study. The palatability of the different processed meals may be different in other species such as HSB but we did not test it due to limited test ingredient availability.

The protein ADC of the reference diet used in the RBT digestibility studies (79.2%) was lower than what was reported (85%), by Barrows et al. (2015). The difference could be because the diets in the present study were cold-pressed not cook-extruded. Another reason could be due to the inclusion of 2% Carboxymethyl cellulose (CMC) to improve the binding of ingredients to produce stable pellets since the diets were not cook-extruded to enable starch gelatinization that improves binding of ingredients in diets (Thomas and

Vander Poel 1996). The protein ADC of carinata meals was generally higher than that of camelina meals because carinata meals generally had lower fiber content, resulting in lower fiber contents of the diets (Tables 3.7 and 3.8). Generally, HSB digested more carinata and camelina meals than RBT, which may be due to a higher fiber in RBT diets due to added CMC or a better tolerance of carinata and camelina meals by HSB than RBT. The reduction in the protein ADC of some AC meals would be due to an increase in the crude fiber content of the diets. Increased crude fiber in the diets generally had less effect on protein ADCs of camelina than carinata meals.

Gross energy ADC of most unconverted carinata and camelina meals associated with protein ADC. It is not clear why gross energy ADC of CPSE camelina was low. Gross energy ADC would be expected to reduce with increased fiber in the diets because fiber is not digested by fish (NRC 2011). However, gross energy ADC was not affected by AC in all camelina meals and in CPAC and CPEXSEAC carinata meals, despite the increase in fiber with AC.

Extrusion mechanically disrupts the lignocellulose structure of plant cell walls (Chinnadurai et al. 2008) which results in improved nutrient ADCs of plant meals due to better accessibility of nutrients by enzymes. However, mixed effects of EX on protein and energy ADCs have been reported. Improved protein ADCs with EX were reported by Booth et al. (2000), and Cheng and Hardy (2003). Extrusion was found to have no effect on protein ADCs by Wilson and Poe (1985), Sorensen et al. (2002), and Barrows et al. 2007. Improved energy ADCs with EX were reported by Wilson and Poe (1985) and Stone et al. (2005). Therefore, the effects of EX on protein and energy ADCs are based on the nature of the ingredients and diet formulations (NRC 2011).

In RBT, protein and energy ADCs reported in other SE oil seed meals are 89 and 77% for dehulled soybean meal, 75 and 49% for cotton seed meal, 75 and 55% for canola meal, and 70 and 34% for linseed meal (Gaylord et al. 2008). Burel et al. (2000b) reported protein and energy ADCs of 90.9 and 88.5%, respectively, for SE rapeseed meal. Most of the different processed carinata meals had a higher or similar protein and energy ADCs than cotton seed meal, canola meal, and linseed meal. However, only a few processed forms of carinata meal had a protein ADC higher than that of soybean or rapeseed meals and all the carinata meals had lower energy ADCs than that of soybean or rapeseed meal. All camelina meals had lower protein and energy ADCs than soybean meal or rapeseed meal. Most of the protein ADCs of camelina meals were similar or higher than those obtained for cotton seed meal, canola meal, and linseed meal. Most of the energy ADCs of camelina meals were close to that of canola seed meal but higher than those of cotton seed meal and linseed meal.

In HSB, some of the reported protein ADCs for solvent extracted oil seed meals are 80% (Sullivan and Reigh 1995) and 86% (Barrows et al. 2015) for soybean meal; 84% (Sullivan and Reigh 1995) and 75% (Barrows et al. 2015) for cotton seed meal; 43% (Gaylord et al. 2004) and 80% (Barrows et al. 2015) for canola meal; and 66% for linseed meal (Barrows et al. 2015). The protein ADC of CPSE carinata meal (98.9%) was better than those of the SE seed meals above. However, CPSE camelina meal (73.6%) had a better protein ADC than only linseed meal.

Conclusion

De-oiled (<1% oil) carinata and camelina meals are high in protein (>46%) compared to most oil seeds used in fish feeds (NRC 2011). However, the meals contain

high GLS (22.5-24.2 $\mu\text{moles/g}$) and crude fiber (7.1-12.6%) contents, hence the tolerance of these meals in piscivorous fishes is low. Determining the best processing methods that yield desirable properties (e.g., high protein, high palatability and ADCs, low GLS and crude fiber contents) will be necessary to adopt these meals as aquafeed ingredients. Based on the results of the present study, CPSEAC would be preferred because the meals produced were higher in protein, lower in soluble carbohydrate, generally higher in essential amino acids and lower in GLS than unconverted meals although higher in crude fiber. The high crude fiber contents of CPSEAC meals generally reduced their protein ADC but the moderate protein ADC can be compensated by high nutrient intake of CPSEAC meals that are low in GLS and / or GLS breakdown products and other soluble ANFs such as sinapine. Generally, carinata meals would be preferred to camelina meals due to a better essential amino acid profile, and higher protein content and ADC. Carinata meals also generally contained lower fiber than the camelina counterparts. Further research is needed to reduce the crude fiber content by pre-digestion of complex carbohydrates with a combination of carbohydrases prior to AC.

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Table 3.1. Proximate composition (dry basis) of processed carinata and camelina meals^a.

Seed Meal (Processing) ^a	Dry Matter (%)	Crude Protein (%)	Crude Lipid (%)	Crude Fiber (%)	Crude Ash (%)	NFE ^b
<i>Carinata</i>						
Whole seed	93.2	28.4	42.8	9.2	4.4	8.4
CP ^a	93.6	45.4	7.8	6.5	7.3	26.6
CPEX ^a	93.8	45.1	7.7	6.8	7.4	26.8
CPSE ^a	93.5	48.2	0.5	6.4	7.8	30.6
CPEXSE ^a	87.5	49.0	0.6	7.1	7.9	22.9
CPAC ^a	92.7	49.1	6.9	13.3	4.4	19.0
CPEXAC ^a	94.8	49.7	12.1	13.2	4.3	15.5
CPSEAC ^a	93.6	56.9	0.6	14.0	5.1	17.0
CPEXSEAC ^a	95.1	55.8	0.5	12.7	4.9	21.2
<i>Camelina</i>						
Whole seed	92.8	28.0	36.6	9.7	3.7	14.8
CP ^a	93.6	42.4	7.5	12.0	5.9	25.8
CPEX ^a	93.6	43.1	7.3	13.9	5.9	23.4
CPSE ^a	93.7	47.1	0.1	12.6	6.4	27.5
CPEXSE ^a	93.1	46.6	0.3	12.8	6.5	26.9
CPAC ^a	93.0	40.9	8.2	22.3	3.2	18.4
CPEXAC ^a	92.0	49.2	5.8	18.9	4.0	14.1
CPSEAC ^a	93.2	53.1	0.7	18.5	4.6	16.3
CPEXSEAC ^a	94.2	53.6	1.48	22.1	5.9	11.1

^aProcessing methods were cold press (CP), extrusion (EX), solvent extraction (SE), aerobic conversion (AC) and sequential combination of the four methods; ^bNFE = Nitrogen free extract = 100 - (moisture + crude protein + crude + lipid + crude fiber + ash).

Table 3.2. Mineral composition (dry basis) of processed carinata and camelina meals^a.

Meal	Macro minerals						Micro minerals			
	P (%)	K (%)	Ca (%)	Mg (%)	Na (%)	S (%)	Zn (ppm)	Mn (ppm)	Cu (ppm)	Fe (ppm)
<i>Carinata</i>										
Whole seed	0.85	1.01	0.44	0.34	ND	0.95	42.45	33.20	5.72	112.52
CP ^a	1.27	1.54	0.62	0.55	0.00	1.65	71.27	50.30	8.29	161.14
CPEX ^a	1.26	1.53	0.61	0.54	0.01	1.62	70.56	49.28	8.46	164.81
CPSE ^a	1.30	1.67	0.68	0.58	0.01	1.72	82.00	53.01	20.71	165.94
CPEXSE ^a	1.22	1.53	0.62	0.54	0.01	1.59	74.42	49.08	16.98	160.82
CPAC ^a	0.92	0.64	0.46	0.26	0.03	1.47	58.50	28.33	10.37	222.01
CPEXAC ^a	0.92	0.63	0.43	0.25	0.03	1.39	58.01	46.82	16.68	212.55
CPSEAC ^a	0.98	0.76	0.50	0.30	0.04	1.61	65.90	32.82	13.63	262.23
CPEXSEAC ^a	0.97	0.69	0.51	0.29	0.08	1.52	68.66	32.53	16.71	519.27
<i>Camelina</i>										
Whole seed	0.78	0.86	0.26	0.34	ND	0.61	42.15	23.15	6.74	95.51
CP ^a	1.10	1.32	0.36	0.50	<0.0015	0.99	67.70	34.73	9.79	133.42
CPEX ^a	1.10	1.37	0.37	0.51	<0.0015	1.00	67.73	35.06	10.32	140.56
CPSE ^a	1.15	1.43	0.42	0.53	0.01	1.08	74.83	37.43	12.06	149.92
CPEXSE ^a	1.15	1.43	0.41	0.54	0.01	1.06	74.67	37.95	11.45	158.30
CPAC ^a	0.52	0.60	0.29	0.26	0.05	1.24	34.49	18.18	18.52	125.70
CPEXAC ^a	0.90	0.61	0.26	0.25	0.05	1.48	46.33	19.55	17.10	217.60
CPSEAC ^a	0.95	0.77	0.29	0.30	0.05	1.62	51.94	31.40	16.12	168.78
CPEXSEAC ^a	1.18	0.53	0.65	0.41	0.12	1.04	96.70	46.41	19.47	432.17

^aProcessing methods were cold press (CP), extrusion (EX), solvent extraction (SE), aerobic conversion (AC) and sequential combination of the four methods; ^bMacro minerals analyzed were phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), Sodium (Na), sulfur (S). Micro minerals included zinc (Zn), manganese (Mn), copper (Cu) and iron (Fe).

Table 3.3. Amino acid profile (g/100g, dry basis) of processed carinata meals in comparison to solvent extracted (SE) soybean meal (SBM)^a.

Amino acid	CP ^a	CPEX ^a	CPSE ^a	CPEXSE ^a	CPAC ^a	CPEXAC ^a	CPSEAC ^a	CPEXSEAC ^a	SE-SBM ^b
Essential									
Arginine	3.38	3.40	3.55	3.96	3.64	3.46	3.97	3.75	3.23
Histidine	1.32	1.33	1.37	1.54	1.40	1.35	1.53	1.46	1.17
Isoleucine	1.69	1.69	1.83	2.03	2.11	2.15	2.46	2.25	1.99
Leucine	3.08	3.10	3.29	3.70	3.97	3.85	4.43	4.10	3.24
Lysine	2.46	2.47	2.53	2.81	2.75	2.70	2.92	2.85	2.83
Methionine	0.86	0.86	0.89	1.01	1.02	0.97	1.12	1.03	0.61
Phenylalanine	1.77	1.79	1.90	2.13	2.31	2.26	2.52	2.37	2.18
Threonine	1.76	1.77	1.84	2.08	2.21	2.07	2.40	2.22	1.73
Tryptophan	0.66	0.67	0.72	0.78	0.67	0.67	0.70	0.67	0.61
Valine	2.09	2.09	2.28	2.52	2.63	2.67	3.06	2.81	2.40
Conditionally essential^c									
Cysteine	1.24	1.25	1.28	1.42	1.15	1.09	1.36	1.21	0.70
Tyrosine	1.10	1.11	1.20	1.32	1.51	1.42	1.67	1.53	1.69
Non-essential									
Alanine	1.84	1.87	1.96	2.21	2.29	2.20	2.55	2.39	-
Aspartic acid	2.81	2.83	3.02	3.37	3.56	3.34	3.87	3.70	-
Glutamic acid	7.77	7.80	8.36	9.42	7.53	7.36	8.84	8.32	-
Glycine	2.12	2.15	2.26	2.55	2.52	2.39	2.81	2.62	-
Proline	2.83	2.84	2.99	3.29	2.90	2.83	3.40	3.12	-
Serine	1.52	1.55	1.54	1.76	1.90	1.76	2.02	1.85	-
Amino acid-like									
Hydroxyproline	0.32	0.34	0.35	0.38	0.47	0.46	0.49	0.44	-
Ornithine	0.02	0.05	0.03	0.04	0.07	0.09	0.06	0.06	-
Taurine	0.11	0.10	0.11	0.13	0.14	0.11	0.13	0.13	-
Totals									
Analyzed protein	45.35	45.14	48.24	48.96	49.11	49.69	56.92	55.73	-
Non-protein nitrogen ^d	4.74	4.21	5.07	0.68	2.58	4.71	4.80	7.05	-

^aProcessing methods were cold press (CP), extrusion (EX), solvent extraction (SE), aerobic conversion (AC) and sequential combinations of the four methods; ^bSE-SBM was 44% crude protein and 1.5% lipid (NRC 2011); ^cConditionally essential amino acids are made from essential amino acids;

^dNon protein nitrogen = analyzed crude protein of the meals (Table 3.1) – \sum (amino acids as a percentage of the seed meal).

Table 3.4. Amino acid profile (g/100g, dry basis) of processed camelina meals in comparison to solvent extracted (SE) soybean meal (SBM)^a.

Amino acid	CP ^a	CPEX ^a	CPSE ^a	CPEXSE ^a	CPAC ^a	CPEXAC ^a	CPSEAC ^a	CPEXSEAC ^a	SE-SBM ^b
Essential									
Arginine	3.53	3.60	3.76	3.71	1.65	3.80	4.14	3.20	3.23
Histidine	1.06	1.07	1.14	1.12	0.82	1.23	1.30	1.11	1.17
Isoleucine	1.54	1.62	1.72	1.72	1.76	2.04	2.16	2.26	1.99
Leucine	2.88	2.91	3.11	3.10	3.01	3.68	3.95	4.05	3.24
Lysine	2.13	2.19	2.25	2.14	2.07	2.50	2.58	2.73	2.83
Methionine	0.76	0.78	0.82	0.82	0.67	0.87	0.93	0.96	0.61
Phenylalanine	1.79	1.83	1.94	1.94	1.86	2.31	2.47	2.41	2.18
Threonine	1.77	1.77	1.86	1.86	1.85	2.10	2.26	2.24	1.73
Tryptophan	0.62	0.45	0.60	0.54	0.24	0.52	0.53	0.47	0.61
Valine	2.16	2.27	2.39	2.39	2.49	2.80	3.01	3.10	2.40
Conditionally essential^c									
Cysteine	0.95	0.93	1.01	1.00	0.77	1.06	1.20	0.75	0.70
Tyrosine	1.11	1.12	1.21	1.18	1.20	1.35	1.48	1.45	1.69
Non-essential									
Alanine	1.94	1.97	2.09	2.08	2.21	2.36	2.58	2.82	-
Aspartic acid	3.53	3.57	3.77	3.76	3.33	4.00	4.44	4.39	-
Glutamic acid	6.73	6.83	7.43	7.33	3.85	7.20	8.18	6.28	-
Glycine	2.21	2.23	2.37	2.36	2.32	2.55	2.75	2.68	-
Proline	2.24	2.29	2.44	2.40	1.98	2.60	2.87	2.33	-
Serine	1.72	1.66	1.71	1.70	1.72	2.01	2.17	2.05	-
Amino acid-like									
Hydroxyproline	0.49	0.49	0.50	0.50	0.75	0.63	0.58	0.55	-
Ornithine	0.06	0.03	0.03	0.04	0.53	0.06	0.15	0.16	-
Taurine	0.10	0.10	0.11	0.12	0.09	0.11	0.10	0.10	-
Totals									
Analyzed protein	42.44	43.07	47.08	46.58	40.86	49.15	53.08	53.63	-
Non-protein nitrogen ^d	3.29	3.50	4.94	4.94	6.29	3.52	3.49	7.78	-

^aProcessing methods were cold press (CP), extrusion (EX), solvent extraction (SE), aerobic conversion (AC) and sequential combinations of the four methods; ^bSE-SBM was 44% crude protein and 1.5% lipid (NRC 2011); ^cConditionally essential amino acids are made from essential amino acids; ^dNon protein nitrogen = analyzed crude protein of the meals (Table 3.1) – \sum (amino acids as a percentage of the seed meal).

Table 3.5. Gross energy, pH, glucosinolate (GLS) and phytic acid (dry basis) of processed carinata and camelina meals^a.

Seed Meal (Processing) ^a	Carinata				Camelina			
	Gross Energy (Kcal/g)	pH	GLS (μ moles/g)	Phytic acid (g/100g)	Gross Energy (Kcal/g)	pH	GLS (μ moles/g)	Phytic acid (g/100g)
CP ^a	5.3	5.9	25.4	3.6	5.1	6.2	26.6	3.0
CPEX ^a	5.0	6.0	23.8	3.7	4.7	6.1	27.2	3.0
CPSE ^a	4.2	5.8	23.7	4.0	4.5	5.9	27.6	2.9
CPEXSE ^a	3.8	5.7	22.5	4.6	4.0	5.7	24.2	3.1
CPAC ^a	5.1	3.7	7.7	2.2	5.1	4.9	0.0	0.6
CPEXAC ^a	5.5	3.9	5.8	2.2	5.0	3.3	0.8	2.5
CPSEAC ^a	4.3	4.3	3.8	3.1	4.6	3.4	0.4	3.2
CPEXSEAC ^a	4.2	4.2	5.5	3.0	4.8	5.9	0.3	2.6

^aProcessing methods were cold press (CP), extrusion (EX), solvent extraction (SE), aerobic conversion (AC) and sequential combinations of the four methods.

Table 3.6. Formulation and composition of diets (g/100g, dry basis) used in palatability and digestibility studies of processed carinata and camelina meals in Rainbow Trout and Hybrid Striped Bass^a.

Ingredients	Rainbow Trout		Hybrid Striped Bass	
	Reference	Test	Reference	Test
FM (Menhaden) ^b	55.0	38.5	55	38.5
Carinata/camelina ^c	0.0	30.0	0.0	30.0
Whole wheat flour ^d	26.9	18.1	33.2	23.2
CMC ^e	2.0	1.4	0.0	0.0
Vitamin premix ^f	1.0	0.7	1.0	0.7
Mineral premix ^g	1.0	0.7	1.0	0.7
Stay-C ^h	0.3	0.2	0.3	0.2
Choline (60%) chloride ⁱ	0.3	0.2	0.0	0.0
Fish oil (Menhaden) ^j	13.0	9.1	9.0	6.3
Chromic oxide ^k	0.5	0.5	0.5	0.5
Composition				
Crude protein	43.2	40.3-44.7	45.4	43.6-47.9
Crude lipid	20.7	14.6-18.1	17.9	12.7-16.2
Crude fiber	2.6	3.8-8.5	0.7	2.5-7.1
Ash	15.4	11.7-13.2	15.5	11.9-13.3
NFE	19.8	17.9-23.0	19.0	16.6-22.5
Gross energy (GE, MJ/g)	21.1	19.5-21.2	20.7	19.3-20.9
Protein:Energy (g/MJ)	20.5	20.6-22.9	21.9	22.6-24.8

^aThe reference diets were based on USDA grain project reference diet 1 for Rainbow Trout but with slight modifications. The reference diets contained 55 % fish meal. The test diets were formulated by substituting 30% of the reference diets with processed carinata or camelina seed meals which included cold pressed (CP), cold pressed and extruded (CPEX), cold pressed and solvent extracted (CPSE), cold pressed, extruded and solvent extracted (CPEXSE) carinata or camelina seed meals and their aerobically converted (AC) products (CPAC, CPEXAC, CPSEAC and CPEXSEAC, respectively).; ^bSpecial select, Omega protein corporation, Houston, TX; ^cAgrisoma, Quebec, Canada (carinata) and Willamette bioprocessors, Oregon (Camelina); ^dRed hard winter wheat, Bob's Red Mill, Milwaukie, Oregon; ^eAkzo Nobel Functional Chemicals BV, Amersfoort, Netherlands; ^fARS 702 vitamin premix, Nelson and Sons, Murray, UT; ^gARS 640 trace mineral premix; ^hArgent laboratories, Redmond, WA; ⁱBiochem Corporation, New Hampton, NY; ^jVirginia Prime Gold, Omega Protein, Houston, TX; ^kFisher Scientific, Pittsburg, PA.

Table 3.7. Dietary crude fiber and apparent digestibility coefficients (ADCs) of protein and gross energy for processed carinata and camelina meals in Rainbow Trout^a.

Seed Meal (Processing) ^a	Carinata			Camelina		
	Crude Fiber (%) ^b	ADCs		Crude Fiber (%) ^b	ADCs	
		Protein (%)	Gross Energy (%)		Protein (%)	Gross Energy (%)
Reference diet	2.58	79.2	77.7	2.58	79.2	77.7
CP ^a	3.81	84.3	56.5	5.46	65.1	38.7
CPEX ^a	3.90	89.0	65.9	6.03	75.4	52.4
CPSE ^a	3.78	92.4	65.9	5.64	67.6	32.4
CPEXSE ^a	3.99	86.1	50.2	5.70	87.0	52.8
CPAC ^a	5.85	81.9	66.6	8.55	76.0	64.3
CPEXAC ^a	5.82	73.9	58.8	7.53	68.4	52.5
CPSEAC ^a	6.06	78.4	54.4	7.41	76.1	57.7
CPEXSEAC ^a	5.67	87.1	60.7	8.49	74.1	58.3

^aProcessed carinata and camelina included cold pressed (CP); cold pressed and extruded (CPEX); cold pressed and solvent extracted (CPSE); cold pressed, extruded and solvent extracted (CPEXSE) and their aerobically converted (AC) products (CPAC, CPEXAC, CPSEAC and CPEXSEAC, respectively); ^bDietary crude fiber was calculated based on the crude fiber composition of dietary ingredients. Carboxymethyl cellulose was 100% crude fiber, whole wheat flour was 3.42% and the crude fiber contents of processed carinata and camelina seed meals are in Table 3.1.

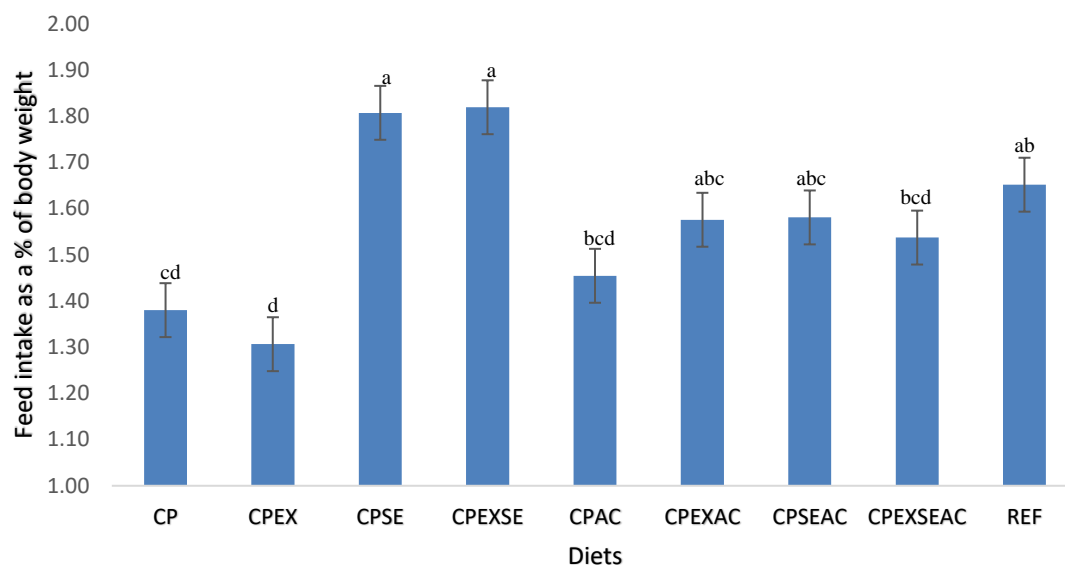
Table 3.8. Dietary crude fiber and apparent digestibility coefficients (ADCs) of protein for processed carinata and camelina meals in Hybrid Striped Bass^a.

Seed Meal (Processing) ^a	Carinata		Camelina	
	Crude Fiber (%) ^b	Protein ADC (%)	Crude Fiber (%) ^b	Protein ADC (%)
Reference diet	0.7	76.1	0.7	76.1
CP ^a	2.53	81.9	4.13	75.4
CPEX ^a	2.53	89.3	4.73	72.3
CPSE ^a	2.43	98.9	4.33	70.5
CPEXSE ^a	2.63	98.3	4.33	82.0
CPAC ^a	4.53	78.6	7.23	68.4
CPEXAC ^a	4.53	85.5	5.23	87.0
CPSEAC ^a	4.73	79.0	6.13	72.8
CPEXSEAC ^a	4.33	83.9	7.13	92.5

^aProcessed carinata and camelina included cold pressed (CP); cold pressed and extruded (CPEX); cold pressed and solvent extracted (CPSE); cold pressed, extruded and solvent extracted (CPEXSE) and their aerobically converted (AC) products (CPAC, CPEXAC, CPSEAC and CPEXSEAC, respectively).

^bDietary crude fiber was calculated based on the crude fiber composition of dietary ingredients. Whole wheat flour was 3.42% and the crude fiber contents of processed carinata and camelina seed meals are in Table 3.1.

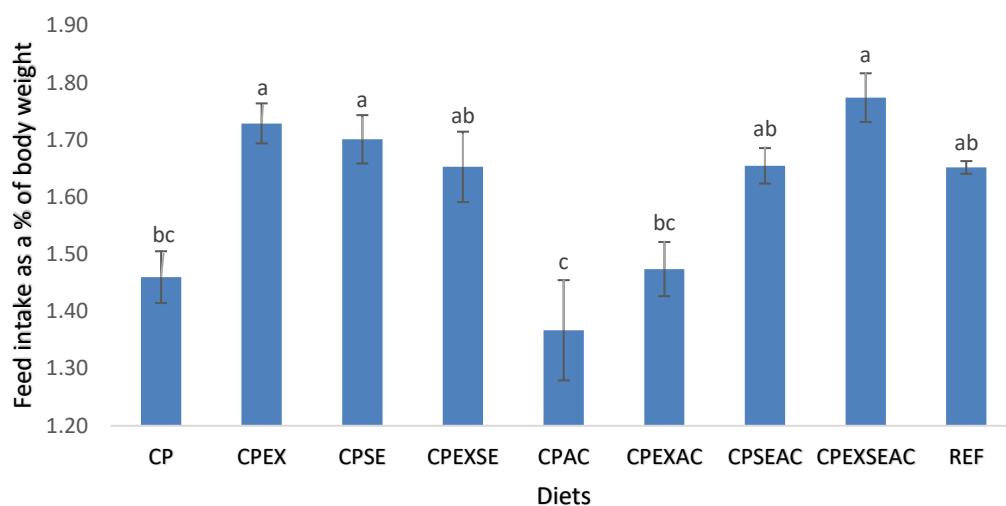
Figure 3.1. Palatability (means \pm SE) of diets containing processed carinata meals in Rainbow Trout^a.



^aPalatability of diets containing 30 % processed carinata seed meals fed for seven days. The diet amounts were divided by fish biomass per tank for comparison across treatments. Processed carinata included cold pressed (CP); cold pressed and extruded (CPEX); cold pressed and solvent extracted (CPSE); cold pressed, extruded and solvent extracted (CPEXSE) and aerobically converted (AC) products (CPAC, CPEXAC, CPSEAC and CPEXSEAC).

*Means with the same letters are not significantly different.

Figure 3.2. Palatability (means \pm SE) of diets containing processed camelina meals in Rainbow Trout^a.



^aPalatability of diets containing 30 % processed carinata seed meals fed for seven days. The diet amounts were divided by fish biomass per tank for comparison across treatments. Processed camelina included cold pressed (CP); cold pressed and extruded (CPEX); cold pressed and solvent extracted (CPSE); cold pressed, extruded and solvent extracted (CPEXSE) and their aerobically converted (AC) products (CPAC, CPEXAC, CPSEAC and CPEXSEAC).

*Means with the same letters are not significantly different.

CHAPTER 4

DIETARY TOLERANCE OF GLUCOSINOLATES FROM CARINATA *Brassica carinata* BY HYBRID STRIPED BASS *Morone chrysops* ♀ X *M. saxatilis* ♂

Abstract

Two experiments were conducted to determine the maximum glucosinolate (GLS) and sinapine concentrations from cold-pressed carinata meal (CPCM) containing 61.2 μ moles of GLS and 6.07 mg of sinapine/g of meal that could be tolerated in diets of Hybrid Striped Bass *Morone chrysops* ♀ X *M. saxatilis* ♂. Based on reported low tolerance of GLS in Rainbow Trout *Oncorhynchus mykiss* (1.4 μ moles of GLS/g of diet), diets in experiment 1 were formulated to provide 0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 μ moles of GLS/g of feed ingredients by including 0, 1.63, 2.45, 3.27, 4.08, 4.90 6.53 and 8.16% of CPCM in diets, respectively. Analyzed GLS and sinapine concentrations after diet manufacture were ≤ 2.71 μ moles and 0.181mg/g of diet, respectively. GLS breakdown products were not detected. The concentrations of GLS and sinapine or other antinutrients in CPCM did not reduce feed consumption, utilization and growth in experiments 1, resulting in similar concentrations of thyroxines and consequently no effect on deiodinase enzymes.

In experiment 2, diets were formulated to provide 0, 6.12, 12.24 and 18.36 μ moles of GLS/g of feed ingredients by including 0, 10, 20 and 30% of CPCM in diets, respectively. Analyzed GLS concentrations were 0, 2.65, 5.58 and 9.52 μ moles of GLS/g of diet, respectively. Sinapine concentrations were 0, 0.31, 0.54 and 0.75 mg/g of diet, respectively. Concentrations of GLS ≥ 5.58 μ moles and sinapine ≥ 0.54 mg/g of diet reduced feed consumption, utilization and growth. Fish response to GLS and / or GLS

breakdown products in carinata is likely due to reduced diet palatability rather than impairment of thyroid function. Significant consumption of CPCM would be required to impair thyroid metabolism. In addition, for reduction in growth to be attributed to impairment of thyroid metabolism, a similar consumption of diets should be achieved first because reduced feed intake/starvation reduces thyroxine production.

Introduction

Glucosinolates (GLS) are produced by plants of family *Brassicaceae* as a deterrent to herbivores and pathogens (Redovnikovic et al. 2008). About 120 different GLS have been reported (Halkier and Gershenzon 2006); however, a single plant contains only a portion of those GLS in higher concentrations (Redovnikovic et al. 2008). Plants store GLS separate from myrosinase, a thioglucosidase enzyme and the two come in contact when their compartments are broken by wounding, insect or pathogen attack, producing hydrolysis products that depend on the structure of GLS side chains, plant species and reaction conditions (Bones and Rossiter 1996; Rask et al. 2000). GLS hydrolysis products include thiocyanates, isothiocyanates, nitriles, and oxazolidinethione (Tripathi and Mishra 2007). Nitriles are neurotoxic (Balbuena and Llorens 2003). Isothiocyanates are bitter (Mithen et al. 2000). Thiocyanates and oxazolidinethione interfere with thyroid metabolism, reducing thyroxine production (Wallig et al. 2002). Other reported negative effects of GLS breakdown products in animals are mutagenicity, hepatotoxicity and nephrotoxicity (Zang et al. 1999; Tanii et al. 2004).

Negative effects of GLS and / or their breakdown have been reported in fish (Yurkowski et al. 1978; Hardy and Sullivan 1983; Letherland et al. 1987; Burel et al. 2000a, b and 2001), limiting inclusion of GLS containing ingredients in fish diets.

Tripathi and Mishra (2007) recommended 1.4 μ moles of GLS/g of diet from rapeseed meal (RSM) *Brassica napus*, as the upper safe level for Rainbow Trout (RBT) *Oncorhynchus mykiss*, because that concentration reduced triiodothyronine (T₃) and growth in the study by Burel et al. (2000a). In red Seabream *Pagrus auratus*, GLS contents of up to 2.18 μ moles /g of diet from canola meal did not reduce feed intake or growth (Glencross et al. 2004a, b). In Turbot *Psetta maxima*, 12.4 μ moles of GLS and estimated 4.4 μ moles of GLS breakdown products /g of diet containing heated RSM, reduced T₃ concentration and feed intake (Burel et al. 2000b). However, GLS concentrations of 11.6 μ moles/g of diet from un-heated RSM did not affect thyroxine (T₄) or T₃ but reduced fish growth due to low feed intake. Fish respond to low thyroxines by altering the activities of deiodinase enzymes (Burel et al. 2000a, b and 2001).

GLS contained in the diets used in the above studies were from RSM or canola meals that contain a high percentage (>54%) of progoitrin (Schone et al. 2001; Yasumoto et al. 2010, Ban et al. 2017) and no sinigrin (Ban et al. 2017) compared to other *Brassica* species such as *B. juncea* that contains ~71% sinigrin and negligible progoitrin (Merah 2015) and *B. carinata* that contains 56.6-95.0% sinigrin (Warwick et al. 2006; Ban et al. 2017) and <2% progoitrin (Ban et al. 2017). Goitrin, the major breakdown product of progoitrin, is nonvolatile (Yasumoto et al. 2010) and will likely be contained in meals after wet processing or diets after manufacture. However, allyl isothiocyanate, the major breakdown product of sinigrin, is volatile (Dai and Lim 2014) and most likely can be removed in meal treatment or diet manufacturing processes. Therefore, GLS and / or

GLS breakdown products from other *Brassica* species other than *B. napus* containing low progoitrin may affect diet palatability, with no significant effect on throxine production.

Carinata *Brassica carinata* is undergoing genetic modification to increase its seed oil and erucic acid contents for jet fuel production (Taylor et al. 2010), which may increase protein (>40%) contents of de-oiled (<1%) meals but also likely to alter the composition of crude fiber (>7.2% of meal, Getinet et al. 1996), sinapine (18.3 mg/g of meal, Wang et al. 1998) and GLS (87.6-138.7 μ moles/g of seed, Warwick et al. 2006) reported before 2000. Dietary tolerances of GLS reported for fish is from RSM GLS for which GLS composition is different from that of carinata. Tolerance of sinapine has not been reported for fish. However, negative effect of sinapine on palatability has been reported (Naczki et al. 1998). Seed meal processing methods to reduce antinutrients vary, but should be aimed at increasing meal intake and utilization efficiency in food animals.

Hybrid Striped (Sunshine) Bass (HSB) *Morone chrysops* ♀ X *M. saxatilis* ♂ is among the top four major food fish produced in the US (USDA-NASS 2012). Limited nutrition studies using GLS containing meals have been conducted on HSB. One study (Webster et al. 2000) included 20% of canola meal in HSB diets containing 27% meat and bone meal and did not observe reduced growth or feed intake when compared to a 30% fish meal reference diet.

Given the lack of information on carinata applications in fish diets, the objective of this study was to determine the maximum tolerable dietary GLS and sinapines from CPCM in HSB.

Materials and Methods

Source of feed ingredients. —Menhaden fish meal (Special Select) and oil (Omega Prime) were obtained from the Omega Protein Corporation (Houston, TX). Cold pressed carinata meal (CPCM) was provided by Dakota Lakes Research Station (Pierre, SD). Corn protein concentrate (Empyreal75) was obtained from Cargill Inc. (Blair, NE). Wheat midds were obtained from Nutra Blend LLC (Neosho, MO). Whole clean wheat was obtained from Ag First Farmers Co-op (Brookings, SD). Poultry by-product meal was obtained from Tyson Foods Inc. (Springdale, AR). Yellow corn gluten was obtained from Kent Nutrition Group (Muscatine, IA). Wheat gluten was obtained from the Manildra Milling Corporation (Leawood, KS). Carboxymethyl cellulose was obtained from Akzo Nobel Functional Chemicals BV (Amersfoort, Netherlands). Vitamin and mineral premixes were specialty blends for fish diets. Stay C was obtained from DSM Jiangshan Pharmaceutical Co. Ltd (Jingjiang, Jiangsu, China). Choline (60%) chloride was obtained from Biochem Corporation (New Hampton, NY). Lysine was obtained from the Anjinomoto Animal Nutrition Group (Chicago, IL). Methionine was obtained from the Adisseo Blue Star Company (North Point Parkway, GA) and soybean oil was provided by South Dakota Soybean Processors (Volga, SD).

Composition of feed ingredients. —Dry matter (AOAC 2006, method 934.01), crude protein (AOAC 2006, method 972.43), crude lipid (AOAC 2006, method 2003.06), crude fiber (AOAC 2006, method 962.09), and ash (AOAC 2006, method 942.05) contents of CPCM were analyzed, as well as moisture and protein contents of other ingredients prior to formulating diets. GLS were analyzed with a Shimadzu (Columbia,

MD) HPLC System using a method developed by Betz and Fox (1994) with slight modifications.

Diet composition. —Eight isonitrogenous and isocaloric diets were formulated for HSB (Table 4.1) for experiment 1. Diets were formulated to contain 42% crude protein and 12% lipid. Diets contained 35.0-38.2% fish meal (FM). CPCM contained 61.2 μ moles of GLS/g of meal as 80.3% sinigrin, 8.3% gluconapin, and 11.4% progoitrin. Based on reported low tolerance of GLS (1.4 μ moles of GLS/g of diet) in RBT by Burel et al. (2000a), CPCM incrementally included in diet formulations at 0, 1.63, 2.45, 3.27, 4.08, 4.90 6.53 and 8.16% resulted in 0.00, 0.71, 1.02, 0.69, 1.39, 1.74, 1.53 and 2.71 μ moles of GLS/g of diet, respectively. Four isonitrogenous and isocaloric diets were formulated for HSB (Table 4.5) for experiment 2. All diets were formulated to contain 42% crude protein and 14% lipid and contained 20% FM and 15% poultry by-product meal. Based on observations from the first experiment, the targeted maximum dietary GLS content of experiment 2 was 18.36 μ moles of GLS/g of feed ingredients. CPCM included in diet formulations at 0, 10, 20 and 30% resulted in 0.00, 2.65, 5.58, 9.52 μ moles of GLS/g of diet, respectively.

Diet manufacture. —All dry ingredients were ground to particles less than 0.8 mm in a Fitzpatrick comminutor mill (Elmhurst, IL) prior to blending all ingredients in a Leland 100DA70 double-action food mixer (Fort Worth, TX). The blended ingredients were cook extruded using an Extru-Tech E325 single screw extruder (Sabetha, KS) to provide 3-mm pellets that were passed through a model HC-1210 drier (Colorado Mill Equipment LLC, Canon City, CO). Dry pellets were vacuum coated with oil in a Phlauer

high performance mixer (A&J Mixing International Inc., Ontario, Canada) and then bagged and frozen at -20°C , pending use.

Fish culture and sampling in experiment 1. —GLS and sinapine tolerance limit studies were conducted under South Dakota State University IACUC protocol #15-064A. Thirty randomly selected fish were stocked in each tank (106L) of a 32-tank recirculating aquaculture system (RAS) and four replicates were used for each diet. The spiny dorsal fin of 10 fish ($22.0 \pm 0.2\text{g}$) was clipped and the remaining 20 fish ($21.5 \pm 0.2\text{g}$) remained unclipped. Temperature, pH and dissolved oxygen were monitored daily using a YSI Professional Plus meter (YSI, Yellow Springs, OH) and averaged (mean \pm SD) $24.2 \pm 1.9^{\circ}\text{C}$, 7.4 ± 0.3 and $5.1 \pm 0.7\text{ mg/L}$, respectively. Unionized ammonia and nitrite were monitored weekly using a Hach DR 2000 spectrophotometer (Hach, Loveland, CO), and averaged $0.003 \pm 0.002\text{ mg/L}$ and $0.05 \pm 0.04\text{ mg/L}$, respectively. Fish were fed twice a day to satiation and feed consumption per individual tank was recorded during the entire study. Three unclipped fish were sacrificed every two weeks. The caudal peduncle was severed and blood was collected in heparinized vacutainers, placed on ice and centrifuged at $3000 \times g$ for 10 mins in a chilled centrifuge to separate plasma. Plasma was immediately frozen at -80°C for later quantification of triiodothyronine (T3) and thyroxine (T4). From the same fish, kidneys, livers and brains were obtained for measuring deiodinase activities I (DI), DII and DIII, respectively. The harvested organs were immediately frozen on dry ice and then stored at -80°C , pending analysis. Clipped fish were weighed after every four weeks to monitor their growth.

After 12 weeks (end of study), in addition to biweekly samplings, the weights of the viscera, livers, spleen and viscera fat were measured. Also, one heparinized micro-

capillary tube was filled with blood for each fish and centrifuged at 10,000 rpm to separate red blood cells (RBCs) for calculation of hematocrit (Hk) values. Additional blood was placed in transformation solution for hemoglobin (Hb) analysis.

Calculation of performance variables. —Growth performance variables were calculated using the following equations;

$$\text{Initial weight/fish (g)} = \frac{\text{Biomass of clipped fish at beginning of study}}{\text{Number of clipped fish at beginning of study}}$$

$$\text{Final weight/fish (g)} = \frac{\text{Biomass of clipped fish at end of study}}{\text{Number of clipped fish at end of study}}$$

$$\text{Weight gain/fish (g)} = \text{Final weight} - \text{initial weight}$$

$$\text{Weight gain/day (g)} = \frac{\text{Weight gain/fish}}{\text{Days of study}}$$

$$\text{Relative growth (\%)} = \frac{\text{Final weight} - \text{initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Specific growth rate (\%)} = 100 \times ((\ln (\text{final weight}) - (\ln (\text{initial}))) / \text{days of growth})$$

$$\text{Survival (\%)} = \frac{\text{Number of fish at end of study}}{\text{Number of fish at start of study}} \times 100$$

$$\text{Feed allowance (g)} = \text{Sum of feed offered from start to end of study.}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Dry feed fed for entire study (g)}}{\text{Biomass gained during study (g)}}$$

Biomass gained was total biomass of clipped, unclipped fish and mortalities in either category.

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Biomass gained during study (g)}}{\text{Protein consumed (g)}}$$

$$\text{Viscerosomatic index (VSI, \%)} = \frac{\text{Weight of viscera (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{\text{Weight of liver (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Spleen somatic index (SSI, \%)} = \frac{\text{Weight of spleen (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Visceral fat somatic index (VFSI, \%)} = \frac{\text{Weight of viscera fat (g)}}{\text{Body weight (g)}} \times 100$$

Blood parameters. — Hematocrit (Hk, %) was calculated as a percentage of red blood cells as:

$$\text{Hk (\%)} = \frac{\text{Length of packed red blood cells (mm)}}{\text{Length of whole blood (mm)}} \times 100$$

Hemoglobin (g/dL) was determined by the cyanmethemoglobin method (Houston 1990).

Eight uL of whole blood was added to 1 mL of transformation solution made by dissolving 1.0 g of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), 0.1 g of potassium cyanide (KCN), and 1.95 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in 1 L deionized water.

Cyanmethemoglobin was used as a standard and optical densities were measured at 540nm in a 96 well plate (200 uL) using a Bio Tek Epoch plate reader (Winooski, VT). The mean corpuscular hemoglobin content (MCHC) was calculated as the hemoglobin (g/dL) content divided by Hk (%).

Plasma was pooled by tank and 50 μL was assayed for T4 or T3 using competitive inhibition (Cat. No. MBS283122) and sandwich (Cat. No. MBS034478) ELISA kits, respectively from MyBiosource Inc. (San Diego, CA). Both ELISAs used horseradish peroxidase (hrp) as the enzyme conjugate and trimethylbenzidine (TMB) as the substrate. The blue color formed after incubation of hrp with TMB, then turned yellow on addition of the stop solution (acid). Optical densities of the solutions were measured at 450 nm using a Bio Tek Epoch plate reader (Winooski, VT).

Tissues (200 mg) obtained from kidneys, livers and brains from three fish were pooled by tank for each sampling time and homogenized in 2mL of phosphate buffered saline (PBS, pH=7.2). The homogenates were centrifuged at 1500 x g in a chilled centrifuge and the supernatant was collected, standardized to 1mg of protein/mL of solution using a bicinchoninic acid (BCA) protein assay kit (Catalogue No. 23225, Fisher Scientific, Rockford, IL) that used serum bovine albumin as a standard. All dilutions were made using PBS. Sandwich ELISA kits MBS067522, MBS041344 and MBS105268 were used to assay DI, DII and DIII, respectively. These kits also used hrp as the enzyme conjugate and TMB as the substrate; analyses were similar to that of T3.

Fish culture and sampling in experiment 2. —Fifteen randomly selected fish (59.1 ± 0.1) were stocked in each tank (106L) of a 30-tank RAS and seven replicates were used for each treatment. Temperature, pH and dissolved oxygen were monitored as in experiment 1 and averaged (mean \pm SD) $26.32 \pm 0.7^{\circ}\text{C}$, 7.4 ± 0.3 and 9.0 ± 0.4 mg/L, respectively. Unionized ammonia and nitrite were also monitored as in experiment 1, and averaged 0.008 ± 0.002 mg/L and 0.08 ± 0.02 mg/L, respectively. Fish were fed twice a day to satiation and feed consumption per tank was recorded during the entire study. Fish were sampled after every three weeks and at the end of the study (8 weeks), all fish in each tank were counted and weighed. Two fish per tank were sacrificed, bled and processed for determination of Hb, Hk and MCHC as in experiment 1. Using the same fish, the viscera, livers, spleens and viscera fat were excised and weighed to calculate their organosomatic indices. Performance metrics were also calculated as in experiment 1 in addition to Fulton's condition factor (K) calculated as;

$$\text{Fulton's (1904) condition factor (K)} = \frac{\text{Weight (g)} \times 100}{\text{Length (cm)}^3}$$

where 100 is a factor required to bring the value of k near unity.

Statistical analysis. —Percentage data from relative growth, VFSI and Hk were \log_{10} transformed; and SGR, VSI and HSI were square-root transformed to achieve normality. All data were analyzed using general linear model in R (R Core Development Team, Vienna, Austria). Tukey's HSD test was used to identify differences among treatment means. Treatment means were considered different at $P < 0.05$.

Results

Experiment 1. —Weight gain ($p=0.24$), relative growth ($p=0.65$), SGR ($p=0.36$), survival ($p=0.22$), feed intake ($p=0.62$), FCR ($p=0.45$) and PER ($p=0.55$) were not affected by CPCPM containing up to 2.71 μ moles of GLS and 0.181mg of sinapine/g of diet, in addition to other antinutrients. VSI ($p=0.63$), HSI ($p=0.70$), SSI ($p=1.00$), VFSI ($p=0.06$), Hk ($p=0.95$), Hb ($p=0.26$) and MCHC ($p=0.36$) were also similar among dietary treatments. GLS contents of up to 2.71 μ moles/g of diet did not alter T_4 ($p=0.44$) or T_3 ($p=0.27$) concentrations in blood of HSB after 12 weeks, resulting in similar DI ($p=0.31$), DII ($p=0.67$) and DIII ($p=0.78$) levels among dietary treatments.

Experiment 2. —Inclusion of 20% of CPCPM providing 5.58 μ moles of GLS and 0.54 mg of sinapine per gram of diet, reduced ($p<0.01$) weight gain. Relative growth ($p<0.01$) and SGR ($p<0.01$) followed the same trend as weight gain. The K value of HSB fed the reference diet was similar to those of HSB fed 10 and 20% CPCPM but higher than that of HSB fed 30% CPCPM. K values of HSB fed 20 and 30% CPCPM were similar. Inclusion of CPCPM in diets of HSB up to 30% and providing up to 9.52 μ moles of GLS and 0.75 mg of sinapine/g of diet, was not lethal. However, fish fed 20 and 30% CPCPM were emaciated and lethargic compared to fish fed the reference diet and 10% CPCPM.

Feed allowance per tank was similar ($p=0.78$) among dietary treatments. However, inclusion of 20 and 30% CPCPM in the diets reduced consumption by 20-50%. Uneaten feed containing 20 and 30% CPCPM would not be accurately quantified. FCR values for fish fed the reference and 10% CPCPM diets were similar and lower ($p<0.01$) than those of diets containing 20 and 30% CPCPM. FCR for the 20% CPCPM dietary treatment was lower than that of the 30% CPCPM treatment. PER of the reference diet and the diet containing 10% CPCPM were similar and higher ($p<0.01$) than those of diets containing 20 and 30% CPCPM, respectively. PER of the diet containing 20% was higher than that of the diet containing 30% CPCPM.

VSI of HSB fed up to 20% CPCPM were similar but higher ($p=0.018$) than that of HSB fed 30% CPCPM. HSI of HSB fed the reference diet was similar to the HSI of HSB fed 10 and 20% CPCPM but higher ($p<0.01$) than the HSI of HSB fed 30% CPCPM. SSI ($p=0.16$), VFSI ($p=0.51$), Hk ($p=0.28$), Hb ($p=0.42$) and MCHC ($p=0.17$) were similar among dietary treatments.

Discussion

Estimated GLS concentrations of 0.0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.12, 12.24 and 18.36 μmoles of GLS/g of feed ingredients were reduced to 0.00, 0.71, 1.02, 0.69, 1.39, 1.74, 1.53, 2.71, 2.65, 5.58 and 9.52 μmoles of GLS/g of diet, respectively (Figure 4.1) by the diet manufacturing process. However, GLS reduction was more variable at lower inclusions of CPCPM. GLS reduction was caused by hydrolysis during diet manufacturing due to water added to the preconditioner with additional steam to hydrate the feed blend.

Similar growth between the reference diet and diets containing up to 10% CPCM was attributed to similar feed consumption. Feed consumption similarly resulted in similar feed conversion and protein efficiency. Reduced growth of HSB fed diets containing 20 and 30% of CPCM was due to reduced feed consumption. A similar feed allowance was used for fish in experiment 2 because the feeding response of diets containing 20 and 30% CPCM was low, with significant consumption sometimes being observed after an hour. Thus, feed was left in the tanks until the next feeding time, at which time any uneaten feed was removed prior to the addition of new feed. Some of the feed had disintegrated, especially for feed left in the tank after the evening feeding, reducing the accuracy of consumption estimates for diets containing 20 and 30% of CPCM. Emaciation and lethargy of HSB fed diets containing 20 and 30% of CPCM is attributed to low feed consumption. Emaciation was more pronounced in HSB fed the diet containing 30% CPCM compared to the 20% CPCM diet and thus the lower K values.

Reduction in feed consumption with increasing CPCM in diets is likely due to the antinutrients in CPCM. All diets used in experiments 1 and 2 contained similar contents of animal meals (35-38) although experiment 1 contained only FM (35.0-38.2%) and experiment 2 contained 20% FM and 15% poultry by-product meal. This difference in the animal meals did not contribute to the observed differences in feed consumption in experiment 2 because poultry by-product meal has been reported to have a similar consumption to FM in HSB (Rawles et al. 2011). Also a similar amount of FM, poultry by-product meal and plant meals were used in all experiment 2 diets. Therefore, GLS, tannins and sinapine likely imparted a bitter and / or astringent taste to CPCM diets, with

bitterness and/ or astringency increasing with increasing CPCM content. All diets containing CPCM contained residual sinigrin, progoitrin, and gluconapin, indicating that the feed manufacturing process did not fully eliminate GLS.

Sinigrin, gluconapin, and progoitrin (Fenwick et al. 1990) are bitter and likely contributed to the bitterness of the diets. GLS breakdown products were not detected in the diets but some myrosinase activity was still present in diets containing CPCM. Myrosinase activity was tested by incubating 10g of ground meals for 24 hours in a buffer solution that traps the resulting GLS breakdown products in dichloromethane. Allyl isothiocyanates were detected but not quantified (Mark Berhow, USDA ARS, personal communication). Therefore, some allyl-isothiocyanates, 3-butenyl isothiocyanates and goitrin may have been formed from hydrolyzed sinigrin, gluconapin and progoitrin, respectively, during consumption, contributing to low palatability because allyl isothiocyanates (VanEtten et al. 1979), 3-butenyl isothiocyanates (Drewnowski and Gomez-Carneros 2000) and goitrin (VanEtten et al. 1979; Fenwick et al. 1990) are bitter and / or pungent. Sinapine (Naczek et al. 1998) and its components (Ismail et al. 1981) are also bitter. Tannins have been reported to impart an astringent taste to foods (Noble 1994, 1998), which may reduce feed consumption. However, they were not quantified in CPCM or diets used in the current study. CPCM likely contained other antinutrients that could have contributed to low palatability of CPCM and the effect on palatability must have increased with increase in CPCM in diets.

In the Burel et al. (2000a) study, consumption of diets containing up to 19.3 μ moles GLS/g from RSM by RBT was low only in the first three weeks of the study and, after that, consumption was similar or higher than the reference diet. Turbot took six

weeks to acclimate to diets containing up to 12.4 $\mu\text{moles GLS/g}$ from RSM, after which feed consumption was similar to that of the reference diet (Burel et al. 2000b). Burel et al. (2001) did not observe any reduction in feed intake when diets containing 7.3 $\mu\text{moles of GLS/g}$ from RSM were fed to RBT. Glencross et al. (2004a, b) did not observe any reduction in feed intake when diets containing up to 2.18 $\mu\text{moles GLS/g}$ of diet from canola meal were fed to Red Seabream *Pagrus auratus*. Consumption of all diets in the current study was relatively similar in the first three weeks of experiment 2 resulting in a similar weight gain, after which decreased consumption occurred for fish fed 20 and 30% CPCM.

The overall difference in consumption of diets containing RSM/canola in the above studies and diets containing CPCM in the current study is likely because of the differences in bitterness of the different GLS components. Sinigrin and gluconapin are reportedly more bitter than progoitrin (Fenwick et al. 1990). The only dietary GLS composition reported from the above studies was by Burel et al. (2000b) and showed that diets contained 38.9-45.6% progoitrin, 14-5-18.6% gluconapin and 0% sinigrin. Diets also contained other GLS such as epi-progoitrin, gluconapoleiferine, glucoalyssine, glucobrassicinapine, sinalbine, 4-hydroxyglucobrassicine, glucosibarine, glucobrassicine, which may not be as bitter as sinigrin and gluconapin. No sinapine concentrations were reported in any of the above studies. Therefore, effect of residual GLS and/ or their breakdown products in RSM/canola on diet palatability is less significant than GLS and/ or their breakdown products in carinata meals. Diets containing 20 and 30% CPCM were less palatable to HSB mainly due to their high sinigrin (~69-74% %) and gluconapin (14.5-19.2%) concentrations and to a small extent, progoitrin (11.4-11.5%).

Survival of all fish in experiment 2 shows that GLS, sinapines and other antinutrients in CPCM have no lethal treatment effect on HSB. Fish in experiment 1 were handled every two weeks to remove three unclipped fish per tank and stress from frequent handling would have resulted in the few mortalities observed. However, survival was not significantly different among dietary treatments. No lethal effect of GLS was also reported by Burel et al. (2000a, b, 2001); Glencross (2004a, b)

HSI decreased with increasing CPCM in experiment 2 and this may be because the low feed intake resulted in breakdown of energy reserves in the liver. Most fish store energy in the liver and liver to body weight indices are normally used to assess overall fish condition (Lambert and Dutil 1997). The calculated combined index of only the intestines and stomachs (2.76%) of HSB fed the diet containing 30% CPCM, was lower than those of HSB fed the reference diet (3.39%) and diets containing 10 (3.38%) and 20% (3.51%) CPCM; and together with the small HSI contributed to the lower VSI of HSB fed 30% of CPCM than those of HSB fed other treatment diets.

Similar feed consumption in studies by Burel et al. (2000a, b, 2001) led them to conclude that it was reduction in T_3 and T_4 induced by GLS breakdown products that was responsible for reduced fish growth. In trying to maintain the required circulating concentrations of T_3 , fish responded by altering activities of deiodinase type I, II and III enzymes in the kidneys, livers and the brain, respectively. Increased DI and DII increase T_3 formation from reverse- T_3 and T_4 and reduced DIII reduces degradation of T_3 to T_2 (Leonard and Visser 1986). All processed RSM in studies by Burel et al. (2000a, b, 2001) had the potential of impairing the thyroid metabolism. Although RSM1 in Burel et al. (2000a, b) contained 5 and 26 μmoles of GLS/g of meal, respectively, meal processing

involved wetting the meals which facilitated hydrolysis of GLS and thus the lower GLS concentration than what was obtained in RSM2 (~40 μ moles GLS/g of meal). Residual myrosinase activity in RSM1 must have been low due to the high temperatures involved in meal processing. However, most goitrin produced by GLS hydrolysis during meal processing must have remained in the meal because goitrin is nonvolatile (Yasumoto et al. 2010). RSM2 was produced by solvent extraction and did not involve any hydration step, accounting for the high GLS contents. Most likely RSM2 retained more myrosinase activity than RSM1 because no heating was involved in meal processing.

Thiocyanates compete with iodine for absorption and vinylloxazolidinethione inhibits the combination of two diiodothyronines (T_2) for form T_4 (Mawson et al. 1994). However, isothiocyanates may not be goitrogenic unless conditions favor their conversion to thiocyanates or vinylloxazolidinethione (Mawson et al. 1994). Therefore, goitrin is a far more potent goitrogen than thiocyanates, and isothiocyanates (Duncan 1991) because its actions cannot be antagonized by iodine (Gaitan 2004). Therefore, the high concentration of progoitrin in RSM2 and likely goitrin in RSM1 accounted for the observed impairment of thyroid metabolism in Burel et al. (2000a, b, 2001) and consequently leading to changes in deiodinase activities. There were no differences in thyroxines or deiodinases activities in experiment 1 because diets containing up to 8.16% CPCM contained ≤ 0.33 μ moles of progoitrin/g of diet and no goitrin, isothiocyanates, or nitriles were detected. Diets in experiment 2 contained ≤ 1.09 μ moles of progoitrin/g of diet and no goitrin, isothiocyanates or nitriles were also detected. Progoitrin concentration in experiment 2 diets were less than the concentrations in diets by Burel et al. (2000b) that lowered T_4 levels and therefore the likelihood of impairing thyroid

metabolism in the current study was low and would only happen if HSB was more sensitive to goitrin than RBT. Reported GLS breakdown products in the diets of Burel et al. (2000b) may not be accurately used to estimate tolerance limits because they were calculated as the difference between GLS content of ingredients before diet manufacture and measured GLS in the diets. With this calculation, it was assumed that all GLS breakdown products remained in the meal, which is not true because breakdown products of other significant GLS in RSM like gluconapin are volatile (Yasumoto et al. 2010).

Thyroxines play a role in growth (Leatherland 1994; Park et al. 2006) by regulating growth hormone metabolism (Melamed et al. 1995). Reductions in T_3 and T_4 have also been reported after reduction in feed intake or short periods of starvation in RBT (Farbridge et al. 1992; Sweeting and Eales 1992; Leatherland and Farbridge 1992) and Tilapia *Oreochromis niloticus* (Toguyeni et al. 1996; Van der Geyten et al. 1998). Based on the above observations, concentrations of thyroxines were not determined in the plasma of experiment 2 fish because they were expected to be low in fish fed diets containing 20 and 30% CPCM due to low feed intake. Therefore, for changes in growth to be attributed to changes in concentrations of thyroxines or growth hormone, diets containing comparable nutrients should be similarly consumed.

Conclusion

Inclusion of >10% CPCM containing >2.65 μ moles of GLS and >0.31mg of sinapine/g of diet was not tolerated by HSB because of reduced diet palatability. Reduced palatability was caused GLS and /or their breakdown products, sinapine and its components, in addition to other antinutrients in CPCM. Variation in inclusion of unprocessed carinata meals in fish diets may vary with concentrations of GLS, sinapine

and other antinutrients in different varieties of carinata since carinata is undergoing genetic modification, which may alter its nutrient and antinutrient composition. Results of this study demonstrate the necessity of processing carinata meals to reduce GLS and/or their breakdown products and sinapines in addition to other antinutrients to improve consumption because palatability not impairment of thyroid metabolism is likely to be the main limitation of using unprocessed carinata meals in fish diets.

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Table 4.1. Formulation of diets (g/100g, dry basis) used in experiment 1 to determine the maximum dietary tolerable glucosinolate (GLS) content from carinata meals of Hybrid Striped Bass^a.

Ingredient	Dietary GLS							
	0.0 ^a	0.71 ^a	1.02 ^a	0.69 ^a	1.39 ^a	1.74 ^a	1.53 ^a	2.71 ^a
Fish meal (menhaden) ^b	38.21	37.87	37.50	37.67	36.95	36.57	35.90	35.02
CPCM ^c	0.00	1.63	2.45	3.27	4.08	4.90	6.53	8.16
Empyreal 75 ^d	10.69	10.42	10.78	10.04	10.33	10.30	10.32	10.39
Wheat midds ^e	24.26	23.40	21.64	22.51	22.06	22.38	20.96	21.20
Whole cleaned wheat ^f	15.00	15.00	15.00	15.00	15.14	14.51	15.10	14.22
Vitamin premix ^e	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Mineral premix ^e	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Stay C ^g	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Choline (60%) chloride ^h	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Fish oil (Menhaden) ^b	8.24	8.08	8.03	7.91	7.84	7.74	7.59	7.41
Composition								
Crude protein (%) ⁱ	42.0	42.0	42.0	42.0	42.0	42.0	42.0	42.0
Crude lipid (%) ⁱ	12.0	12.0	12.0	12.0	12.	12.0	12.0	12.0
Crude fiber (%) ⁱ	3.2	3.3	3.2	3.3	3.4	3.4	3.4	3.5
Ash (%) ⁱ	14.6	14.6	15.0	14.6	14.5	14.5	14.3	14.2
Nitrogen free extract (%) ⁱ	24.3	24.7	24.9	25.2	25.1	25.2	25.5	25.6
Gross energy (GE, MJ/g) ⁱ	17.4	17.5	17.5	17.6	17.7	17.6	17.8	17.8
Protein:Energy (g/MJ) ⁱ	24.1	23.9	23.9	23.8	23.8	23.8	23.6	23.6
Glucosinolates (μmoles/g)								
Progoitrin ^j	0.00	0.08	0.11	0.05	0.16	0.20	0.17	0.33
Sinigrin ^j	0.00	0.36	0.61	0.32	0.92	1.20	0.99	1.96
Gluconapin ^j	0.00	0.27	0.30	0.32	0.31	0.34	0.37	0.42
Total glucosinolates	0.00	0.71	1.02	0.69	1.39	1.74	1.53	2.71
Sinapic acid & its derivatives (mg/g)								
Sinapine ^j	0.00	0.034	0.082	0.094	0.106	0.121	0.142	0.181
sinapoyl glucose ^j	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
sinapic acid ^j	0.00	0.024	0.031	0.033	0.034	0.035	0.038	0.041

^aDietary GLS concentrations were achieved by including 0, 1.63, 2.45, 3.27, 4.08, 4.90 6.53 and 8.16% of cold pressed carinata meal (CPCM) containing 61.2 μmoles of GLS/g of meal in the diets. All diets contained ~36.6-38.2% fish meal (FM), ~42% crude protein and 12% crude lipid. ^bOmega proteins, Houston, TX; ^cDakota Lakes Research Station, SD; ^dCargill, Blair, NE; ^eNutra Blend LLC, Neosho, MO; ^fAg First Farmers Co-op, Brookings, SD; ^gDSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China ^hBiochem Corporation, New Hampton, NY. ^hCalculated based on ingredient composition; ⁱAnalyzed.

Table 4.2. Performance of Hybrid Striped Bass in experiment 1 fed diets with increasing glucosinolate (GLS) contents from carinata meals^a.

Variable	Dietary GLS								Pooled SE	P-values
	0.0 ^a	0.71 ^a	1.02 ^a	0.69 ^a	1.39 ^a	1.74 ^a	1.53 ^a	2.71 ^a		
Initial weight (g)	21.7	21.5	22.3	21.5	21.7	22.1	21.8	22.0	0.12	0.782
Final weight (g)	93.4	87.3	97.3	95.6	93.4	96.7	84.0	88.8	4.0	0.231
Wt. gain (g) ^b	72.0	66.7	75.3	73.9	71.6	75.8	62.5	67.5	3.9	0.243
Wt. gain/ day (g)	0.86	0.80	0.90	0.88	0.85	0.90	0.75	0.80	0.05	0.261
Rel. growth (%) ^c	332.0	327.0	323.0	328.0	311.0	350.0	298.0	339.0	18.2	0.648
SGR (%) ^d	1.75	1.72	1.78	1.77	1.73	1.83	1.61	1.70	0.06	0.361
Survival (%)	97.5	92.5	98.3	100.0	97.5	97.5	98.4	92.5	2.4	0.216
Feed intake/tank (g)	2496	2490	2364	2318	2646	2396	2437	2371	115.7	0.622
FCR ^e	1.39	1.61	1.41	1.39	1.51	1.38	1.54	1.42	0.09	0.446
PER ^f	1.72	1.50	1.73	1.75	1.58	1.75	1.56	1.69	0.11	0.547

^aDietary GLS concentrations were achieved by including 0, 1.63, 2.45, 3.27, 4.08, 4.90 6.53 and 8.16% of cold pressed carinata meal (CPCM) containing 61.2 μ moles of GLS/g of meal in the diets. All diets contained ~36.6-38.2% fish meal (FM), ~42% crude protein and 12% crude lipid. ^bWeight gain/day;

^cRelative growth; ^dSpecific growth rate; ^eFeed conversion ratio; ^fProtein efficiency ratio.

Table 4.3. Organosomatic indices and blood parameters of Hybrid Striped Bass in experiment 1 fed diets with increasing glucosinolate (GLS) contents from carinata meals^a.

Variable	Dietary GLS								Pooled SE	P-values
	0.0 ^a	0.71 ^a	1.02 ^a	0.69 ^a	1.39 ^a	1.74 ^a	1.53 ^a	2.71 ^a		
VSI (%) ^b	10.7	10.8	10.6	10.7	11.4	11.5	11.6	10.9	0.5	0.630
HSI (%) ^c	3.95	3.93	3.62	3.96	4.15	3.82	3.96	3.83	0.2	0.696
SSI (%) ^d	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.01	0.998
VSFI (%) ^e	4.23	4.34	4.55	4.33	4.69	5.21	5.11	4.45	0.2	0.06
Hematocrit (%)	64.8	64.7	66.5	66.7	67.0	65.5	63.8	65.7	3.3	0.946
Hemoglobin (g/dL)	9.9	9.8	9.6	9.3	10.4	9.5	9.0	9.7	0.6	0.261
MCHC (g/dL) ^f	15.5	15.3	14.4	13.9	15.6	14.7	14.1	14.8	1.1	0.362

^aDietary GLS concentrations were achieved by including 0, 1.63, 2.45, 3.27, 4.08, 4.90 6.53 and 8.16% of cold pressed carinata meal (CPCM) containing 61.2 μ moles of GLS/g of meal in the diets. All diets contained ~36.6-38.2% fish meal (FM), ~42% crude protein and 12% crude lipid. ^bViscerosomatic index; ^cHepatosomatic index; ^dSpleen somatic index; ^eVisceral fat somatic index; ^fMean corpuscular hemoglobin content.

Table 4.4. Thyroid hormones and deiodinase activities of Hybrid Striped Bass after 12 weeks in experiment 1 fed diets with increasing glucosinolate (GLS) contents from carinata meals^a.

Variable	Dietary GLS							Pooled SE	P- value
	0.0 ^a	0.71 ^a	1.02 ^a	1.39 ^a	1.74 ^a	1.53 ^a	2.71 ^a		
T ₄ (ng/mL) ^b	10.2	8.2	5.6	12.6	6.9	10.3	16.2	3.5	0.439
T ₃ (ng/mL) ^c	1.9	2.5	2.8	2.8	2.5	2.4	2.3	0.3	0.273
DI (pg/mL/ mg of protein) ^d	141	115	119	142	140	127	105	12.9	0.308
DII (pg/mL/mg of protein) ^e	143	178	146	143	133	98	159	29.8	0.671
DIII (pg/mL/mg of protein) ^f	787	904	849	608	429	801	603	185.6	0.784

^aDietary GLS concentrations were achieved by including 0, 1.63, 2.45, 4.08, 4.90 6.53 and 8.16% of cold pressed carinata meal (CPCM) containing 61.2 μ moles of GLS/g of meal in the diets. All diets contained ~36.6-38.2% fish meal (FM), ~42% crude protein and 12% crude lipid. Samples from fish fed diet GLS 0.69 not analyzed due to kit limitation. Thyroid hormones reported in nanograms and deiodinase activities in picograms. Only results for the last sampling are presented because no differences were detected in preceding samples. ^bTyroxine; ^cTriiodothyronine; ^dDeiodinase activity I; ^eDeiodinase activity II; ^fDeiodinase activity III.

Table 4.5. Formulation of diets (g/100g, dry basis) used in experiment 2 to determine the maximum dietary tolerable glucosinolate (GLS) content from carinata meals of Hybrid Striped Bass^a.

Ingredient	Dietary GLS			
	0.0 ^a	2.65 ^a	5.58 ^a	9.52 ^a
Fish meal (Menhaden) ^b	20.00	20.00	20.00	20.00
CPCM ^c	0.00	10.00	20.00	30.00
Poultry by-product meal ^d	15.00	15.00	15.00	15.00
Wheat midds ^e	17.00	14.81	14.95	13.17
Yellow corn gluten ^f	10.00	7.77	2.80	1.07
Whole cleaned wheat ^g	18.15	16.00	12.33	10.00
Wheat gluten ^h	5.43	3.67	4.00	1.61
CMC ⁱ	1.20	0.90	0.45	0.00
Vitamin premix ^e	1.25	1.25	1.25	1.25
Mineral premix ^e	1.10	0.95	0.80	0.75
Stay C ^j	0.30	0.30	0.30	0.30
Choline (60%) chloride ^k	0.30	0.30	0.30	0.30
Lysine ^l	0.20	0.20	0.20	0.20
Methionine ^m	0.12	0.10	0.12	0.10
Fish oil (Menhaden) ^b	7.00	7.00	7.00	6.25
Soybean oil	2.95	1.75	0.50	0.00
Composition				
Crude protein ⁿ	42.09	42.04	42.32	42.25
Crude lipid ⁿ	14.02	14.03	14.03	14.00
Crude fiber ⁿ	4.21	4.24	4.31	4.23
Ash ^o	10.32	10.67	11.10	11.57
Nitrogen free extract ⁿ	18.93	19.64	18.91	19.47
Gross energy (GE, MJ/g) ⁿ	19.45	19.57	19.52	19.57
Protein:Energy (g/MJ) ⁿ	21.63	21.47	21.67	21.57
Glucosinolates (μmoles/g)				
Progoitrin ^o	0.00	0.28	0.64	1.09
Sinigrin ^o	0.00	1.56	3.87	7.05
Gluconapin ^o	0.00	0.81	1.07	1.38
Total glucosinolates	0.00	2.65	5.58	9.52
Sinapic acid & its derivatives (mg/g)				
Sinapine ^o	0.00	0.31	0.54	0.75
sinapoyl glucose ^o	0.00	0.01	0.03	0.05
sinapic acid ^o	0.00	0.06	0.08	0.10

^aDietary GLS concentrations were achieved by including 0, 10, 20 and 30% of cold pressed carinata meal (CPCM) containing 61.2 μmoles of GLS/g of meal in the diets. All diets contained 10% fish meal and 10% chicken by-product meal; ~42% crude protein and 14% crude lipid. ^bOmega proteins, Houston, TX; ^cDakota Lakes Research Station, SD; ^dTyson Foods Inc., Springdale, AR; ^eNutra Blend LLC, Neosho, MO; ^fKent Nutrition Group, Muscatine, Iowa; ^gAg First Farmers Co-op, Brookings, SD; ^hManildra Milling Corporation, Leawood, KS; ⁱAkzo Nobel Functional Chemicals BV, Amersfoort, Netherlands; ^jDSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China; ^kBiochem Corporation, New Hampton, NY; ^lAnjinomoto Animal Nutrition group, Chicago, IL; ^mAdisseo Blue Star Company, North Point Parkway, GA. ⁿCalculated based on ingredient composition; ^oAnalyzed.

Table 4.6. Performance of Hybrid Striped Bass in experiment 2 fed diets with increasing glucosinolates (GLS) from carinata meals^a.

Variable	Dietary GLS				P-value
	0.0 ^a	2.65 ^a	5.58 ^a	9.52 ^a	
Initial weight (g)	59.19±0.24	58.86±0.19	59.24±0.27	59.00±0.22	0.675
Final weight (g)	144.17±2.10 ^a	142.86±3.02 ^a	125.14±2.59 ^b	106.17±1.26 ^c	<0.001
Weight gain (g)	85.00±1.98 ^a	84.00±2.97 ^a	65.90±2.55 ^b	47.17±1.28 ^c	<0.001
Weight gain/day (g)	1.05±0.02 ^a	1.04±0.04 ^a	0.81±0.03 ^b	0.58±0.02 ^c	<0.001
Relative growth (%)	143.64±3.15 ^a	142.71±4.97 ^a	111.26±4.27 ^b	79.96±2.26 ^c	<0.001
SGR (%) ^b	1.10±0.02 ^a	1.09±0.03 ^a	0.92±0.03 ^b	0.72±0.02 ^c	<0.001
Fulton's K ^c	1.12±0.02 ^{ab}	1.18±0.03 ^a	1.14±0.02 ^{ab}	1.08±0.01 ^b	0.017
Survival (%)	100±0.0	100±0.0	100±0.0	100±0.0	-
Feed allowance/tank (g)	2577.5±47.0	2612.0±52.8	2644.6±37.4	2633.1±55.8	0.778
FCR ^d	2.03±0.07 ^c	2.09±0.09 ^c	2.70±0.10 ^b	3.75±0.14 ^a	<0.001
PER ^e	1.14±0.04 ^a	1.12±0.05 ^a	0.87±0.03 ^b	0.63±0.02 ^c	<0.001

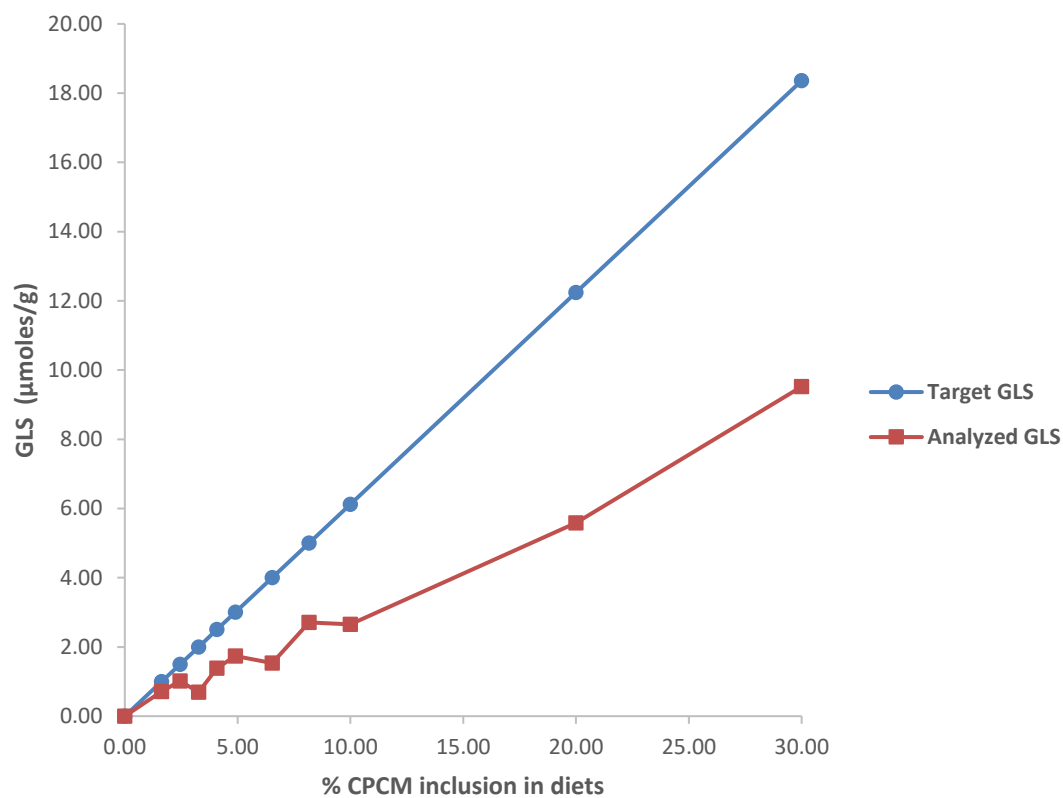
^aDietary GLS concentrations were achieved by including 0, 10, 20 and 30% of cold pressed carinata meal (CPCM) containing 61.2 µmoles of GLS/g of meal in the diets. All diets contained ~36.6-38.2% fish meal (FM), ~42% crude protein and 12% crude lipid. Values are means±standard error; ^bSpecific growth rate; ^cFulton's condition factor; ^dFeed conversion ratio; ^eProtein efficiency ratio.

Table 4.7. Organosomatic indices and blood parameters of Hybrid Striped Bass in experiment 2 fed diets with increasing glucosinolate (GLS) contents from carinata meals^a.

Variable	Dietary GLS				P-value
	0.0 ^a	2.65 ^a	5.58 ^a	9.52 ^a	
VSI (%) ^b	11.52±0.15 ^a	12.00±0.58 ^a	11.76±0.47 ^a	10.19±0.44 ^b	0.018
HSI (%) ^c	2.36±0.09 ^a	2.29±0.21 ^a	1.97±0.09 ^{ab}	1.64±0.05 ^b	<0.001
SSI (%) ^d	0.061±0.003	0.057±0.002	0.06±0.087	0.065±0.003	0.156
VFSI (%) ^e	5.69±0.16	6.27±0.41	6.22±0.48	5.73±0.23	0.505
Hematocrit (%)	57.12±2.02	53.93±1.23	53.78±2.20	51.59±2.43	0.280
Hemoglobin (g/dL)	9.19±0.51	8.86±0.21	8.46±0.23	9.14±0.25	0.417
MCHC (g/dL) ^f	16.26±0.72	16.54±0.30	15.89±0.61	18.04±0.94	0.167

^aDietary GLS concentration were achieved by including 0, 10, 20 and 30% of cold pressed carinata meal (CPCM) containing 61.2 µmoles of GLS/g of meal in the diets. All diets contained ~36.6-38.2% fish meal (FM), ~42% crude protein and 12% crude lipid. Values are means±standard error. ^bViscerosomatic index; ^cHepatosomatic index; ^dSpleen somatic index; ^eVisceral fat somatic index; ^fMean corpuscular hemoglobin content.

Figure 4.1. Glucosinolate (GLS) concentrations before (estimated from cold-pressed carinata meal, CPCM) and after (analyzed) diet manufacturing in glucosinolate tolerance limit studies.



CHAPTER 5

PERFORMANCE OF RAINBOW TROUT *Oncorhynchus mykiss* FED DIETS CONTAINING CARINATA *Brassica carinata* SEED MEALS

Abstract

Carinata *Brassica carinata* is among the oilseed crops for which oil and erucic acid contents are being genetically modified to increase their value for jet-fuel production. Due to the high oil (>40%) content of carinata seeds, the protein content (>40%) of de-oiled carinata meals will be high, providing potential for use in fish diets. Like most plant meals, carinata seed meals contain various antinutrients, but their use in fish diets will be mainly limited by their high contents of crude fiber, glucosinolates and sinapine. We processed hexane extracted carinata seed meal by aerobic conversion (AC) using fungi ssp. followed by a single wash to produce aerobically converted carinata meal (ACCM). We then formulated four low fish meal (FM) diets to contain ~42% protein and 17% lipid, including a fish meal (FM) reference diet (20% FM). In the test diets, ACCM replaced 25, 50 and 75% of FM in the reference diet.

After a 56-day growth period, replacement of more than 25% FM by ACCM, resulted in reduced ($p<0.01$) growth. Reduced growth was partly due to reduced ($p<0.01$) feed consumption in some FM replacement diets, however, no difference in feed consumption was observed between the reference diet and diet with the highest FM replacement. Fulton's condition factor K decreased ($p<0.01$) with increased FM replacement. FCR ($p<0.01$) had an inverse relationship with diet consumption. The trend in FCR was similar to the trend in PER ($p<0.01$). Apparent net protein utilization was lower ($p<0.01$) for the highest FM replacement diet. There was no effect of FM

replacement by ACCM on whole-body composition; viscera, spleen and liver weights. However, visceral fat increased ($p < 0.01$) with increased feed consumption. There were also no effects of replacing FM by ACCM on hematocrit, hemoglobin or mean corpuscular hemoglobin contents. Results of this study show that more than 25% of FM cannot be replaced by ACCM in low FM/animal (20%) diets of RBT and improved utilization of ACCM may require more animal meals in the diets or additional processing of ACCM to improve its feeding value.

Introduction

Rainbow Trout (RBT) *Oncorhynchus mykiss* is one of the most commercially produced food fish in the US (USDA-NASS 2014). Typical commercial RBT feeds contain portions of fish meal (FM) and fish oil in accord with RBT's carnivorous feeding habits. However, stagnating production and competition for FM with other animal feed production sectors have increased FM prices (FAO 2016). Due to the fast growth of the food fish production industry (FAO 2016) and dynamic market, the availability of FM for use in fish feeds is unreliable. Animal meals such as poultry by-product meal, meat and bone meal, blood meal and hydrolyzed feather meal are high in protein (NRC 2011) and may replace much of FM in fish feeds. However, if consumers learn that fish feeds contain such ingredients, fish acceptance may be low (Naylor et al. 2009) due to ethical or religious constraints. Therefore, plant meals are sustainable alternatives to FM use in fish diets providing that they can be mass produced at lower prices than FM and most other animal meals. Also, increasing production of plant meals in the future to meet increasing feedstuff demands is more feasible.

On-going research to replace FM with plant meals has focused mainly on legumes, grains and oilseed meals (Gatlin et al. 2007). Oilseeds such as soybean meal (Gao et al. 2011), rapeseed meal (Burel et al. 2001), canola meal (Slawski et al. 2013), linseed meal (Thiessen 2004), cotton seed meal (Luo et al. 2006), and camelina meal (Hixson et al. 2014), have been used to partially replace FM in RBT diets because of their high protein (>30%) contents (NRC 2011). Different inclusion levels of the above meals are reported in literature based on the type and amount of processing. The amount of FM and /or total animal meals used in FM replacement studies with the above meals may also limit their inclusion levels, as higher inclusion levels may be tolerated at higher levels of FM and / or animal meals in diets, which may not be typical of the current commercial RBT feed formulations. Plant meals also contain a variety of antinutrients that can lower palatability, nutrient intake and utilization at high inclusion levels in fish feeds, which consequently results in reduced growth and may impact health (Francis et al. 2001, Kroghdahl et al. 2010). Plant meals also contain high carbohydrate energy (NRC 2011) which is not well utilized by carnivorous fishes (Wilson 1994; Moon 2001). To increase the acceptability and utilization of plant meals especially by carnivorous fish, plant meals have to be processed to reduce antinutrients to tolerable levels (Hardy 2010).

Carinata *Brassica carinata* seeds are being genetically modified to increase their oil and erucic acid contents (Jadhav et al. 2005, Taylor et al. 2010) to increase their production for biofuel oil. Therefore, the de-oiled meals will be available for use in animal diets. Because carinata seeds contain moderate protein (25.9 to 30.5%, Pan et al. 2012) and high lipid (~40.8%, Xin et al. 2013), the de-oiled meals will be higher in protein (>40%) making them appropriate for use in fish diets, especially in higher

replacements of FM on an equal protein basis. Although the amino acid composition of de-oiled carinata (Table 3.3) is inferior to that of FM, not unlike other plant meals, it is comparable to that of soybean meal (NRC 2011), which is the main plant protein source in fish diets (NRC 2011). De-oiled carinata meal also contains some taurine (Table 3.3) that is conditionally essential for some carnivorous fish such as fast growing RBT fed diets low in FM and high in plant meals (Gaylord et al. 2006). Carinata oil contains 13.7 to 18.9% linoleic acid and 10.2-16.0% linolenic acid (Warwick et al. 2006) that are beneficial to fish based on a species capacity to convert them into highly unsaturated fatty acids (Tocher 2003).

Carinata meal contains glucosinolates (GLS), phytates, (Rakow and Getinet 1998; Pedroche et al. 2004), tannins (Xin et al. 2014), erucic acid (Warwick et al. 2006) and sinapine (Mailer et al. 2008) in addition to non-starch polysaccharides (NSPs) that are inherent to all plant meals as structural components of cell walls (NRC 2011). The inclusion levels of carinata meals in fish diets will mostly be limited by their contents of GLS, sinapine and crude fiber. Therefore, carinata meals need to be processed to increase their feeding value and the initial targets should be the three aforementioned antinutrients. Submerged microbial fermentation occurs in a liquid medium and the water facilitates GLS hydrolysis (Gimsing and Kirkegaard 2009) and solubilization of sinapine (Tan et al. 2011). The enzymes produced by the microbes lower NSPs (Pandey et al. 2000). Solubilized sinapine can be removed in the centrate and isothiocyanates (ITC), formed from GLS hydrolysis, can be evaporated at room temperature (Mari et al. 2008). Any residual ITC and sinapine can be volatilized during drying of the processed meals. The resulting meal should enable more carinata use in fish diets.

Consequently, the objective of this study was to determine how much FM could be replaced by ACCM in diets of RBT containing 20% FM as the only animal protein.

Materials and Methods

Source of and preparation of feed ingredients. —Menhaden fish meal (Special Select) and oil (Omega Prime) were obtained from the Omega Protein Corporation (Houston, TX). Hexane extracted (HE) carinata meal was contributed by Agrisoma Biosciences Inc., Quebec, Canada. Carinata (HE) meal was aerobically converted (AC) using fungi spp. (William. Gibbons, South Dakota State University, personal communication) followed by a single wash to produce aerobically converted carinata meal (ACCM). Yellow corn gluten was obtained from Kent Nutrition Group (Muscatine, IA). Wheat gluten was obtained from the Manildra Milling Corporation (Leawood, KS). Whole clean wheat and sodium bicarbonate were obtained from Ag First Farmers Co-op (Brookings, SD). Brewer's yeast was obtained from Diamond V Mills Inc. (Cedar Rapids, IA). Carboxymethyl cellulose was obtained from Akzo Nobel Functional Chemicals BV (Amersfoort, Netherlands). Vitamin and mineral premixes were specialty blends for fish diets. Stay C was obtained from DSM Jiangshan Pharmaceutical Co. Ltd (Jingjiang, Jiangsu, China). Choline (60%) chloride was obtained from Biochem Corporation (New Hampton, NY). Arginine, cysteine and betaine were obtained from Pure Bulk (Rosenburg, OR). Lysine was obtained from the Anjinomoto Animal Nutrition Group (Chicago, IL). Methionine was obtained from the Adisseo Blue Star Company (North Point Parkway, GA). Taurine was obtained from the Jiangyin Huachang Food Additives (Jiangyin Jiangsu, China). Sodium chloride was obtained from Compass Minerals America Inc. (Overland Park, KS). Potassium chloride was obtained from the

Phibro Animal Health Corporation (Teaneck, NJ). Monocalcium phosphate was obtained from the PCS Sales (Northbrook, IL). Calcium propionate was obtained from Acros Organics (Morris Plains, NJ). Lecithin was obtained from Solae LLC (St. Louis, MO).

Composition of feed ingredients. —Dry matter (AOAC 2006, method 934.01), crude protein (AOAC 2006, method 972.43), crude lipid (AOAC 2006, method 2003.06), crude fiber (AOAC 2006, method 962.09), and ash (AOAC 2006, method 942.05) contents were analyzed for ACCM, as well as; moisture and protein contents of other ingredients prior to formulating diets.

Diet composition and manufacturing. —Four isonitrogenous (~42% crude protein) and isocaloric diets (~5,269 Kcal/kg) were formulated (Table 5.1) for RBT. All diets contained ~17% crude lipid with 3% as lecithin and 11.8-12.5% as fish oil based on FM replacement. The reference diet contained 20% FM and 0% ACCM. Carinata meal incrementally replaced 25, 50 and 75% of FM in the test diets. All dry ingredients were ground to particles less than 0.8 mm in a Fitzpatrick comminutor mill (Elmhurst, IL) prior to blending all ingredients in a Leland 100DA70 double-action food mixer (Fort Worth, TX). The blended ingredients were cook extruded using an Extru-Tech E325 single screw extruder (Sabetha, KS) to provide 3mm pellets that were passed through a model HC-1210 drier (Colorado Mill Equipment LLC, Canon city, CO). Dry pellets were vacuum coated with the remaining oil in a Phlauer high performance mixer (A&J Mixing International Inc., Ontario, Canada), then bagged and frozen at -20⁰ C, pending use.

Fish culture and sampling. — This study was conducted under South Dakota State University IACUC protocol # 16-040A. Twenty fish (19.9±0.09g) were stocked into each tank (106L) of a 32-tank, recirculating aquaculture system (RAS); 10 randomly

selected fish of the same size were euthanized and frozen for later composition analysis. Eight replicates of the reference and each test diet were randomly assigned to the individual tanks. Temperature, pH and dissolved oxygen were monitored daily using a YSI Professional Plus meter (YSI, Yellow Springs, OH) and averaged (mean \pm SD) $15.1\pm0.05^{\circ}\text{C}$, 8.3 ± 0.03 and 6.6 ± 0.05 mg/L, respectively. Unionized ammonia and nitrite were monitored weekly using a Hach DR 2000 spectrophotometer (Hach, Loveland, CO) and averaged (mean \pm SD) 0.026 ± 0.002 mg/L and 0.10 ± 0.01 mg/L, respectively. Fish were fed twice a day to satiation and feed consumption per individual tank was recorded during the entire study. Tank weights were measured every two weeks to monitor growth. At the end of the study (56 days), two fish from each tank were euthanized, weights and lengths were measured, and sampled for blood. One heparinized micro-capillary tube was filled with blood for each fish and centrifuged at 10,000 rpm to separate red blood cells (RBCs) for calculation of hematocrit (Hk) values. Additional blood was placed in the transformation solution for hemoglobin (Hb) analysis. The same fish were necropsied to collect viscera to measure livers, spleens and viscera fat weights and calculation of organo-somatic indices. The remaining fish in each tank were also weighed and counted to determine total tank biomass and survival. Three additional fish were euthanized, frozen and later homogenized to provide a homogenous sample per replicate. The 10 fish frozen at the beginning of the study were also homogenized. These homogenized fish samples were frozen and then freeze dried (Labconco Freezone 2.5 freeze-dryer, Kansas City, MI) for 72 hours. The dried fish samples were finely ground in a coffee grinder, mixed to ensure uniformity and analyzed for protein (AOAC 2006, method 972.43).

Protein concentrations were used to calculate apparent protein deposition (APD) and apparent net protein utilization (ANPU).

Calculation of performance variables. —The growth performance variables were calculated using the following equations:

$$\text{Initial weight/fish (g)} = \frac{\text{Tank biomass at the beginning of study}}{\text{Number of fish in the tank}}$$

$$\text{Final weight/fish (g)} = \frac{\text{Tank biomass at end of study}}{\text{Number of fish in the tank}}$$

$$\text{Weight gain/fish (g)} = \text{Final weight} - \text{initial weight}$$

$$\text{Weight gain/day (g)} = \frac{\text{Weight gain}}{\text{Days of study}}$$

$$\text{Relative growth (\%)} = \frac{\text{Final weight} - \text{initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Specific growth rate (\%)} = 100 \times ((\ln (\text{final weight}) - (\ln (\text{initial}))) / \text{days of growth})$$

$$\text{Fulton's (1904) condition factor (K)} = \frac{\text{Weight (g)} \times 100}{\text{Length (cm)}^3}$$

where 100 is a factor required to bring the value of k near unity

$$\text{Survival (\%)} = \frac{\text{Number of fish at end of study}}{\text{Number of fish at start of study}} \times 100$$

$$\text{Feed consumed (g)} = \text{Sum of feed fed from start to end of study}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Dry feed fed for entire study(g)}}{\text{Wet biomass gained during study (g)}}$$

$$\text{Protein consumed (g)} = \text{Feed consumed (g)} \times \text{percentage of protein in feed}$$

$$\text{Wet biomass gained} = \text{Tank biomass at end of study (g)} + \text{biomass of mortalities during the study (g)}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Wet biomass gained during study(g)}}{\text{Protein consumed (g)}}$$

Protein consumed (g)

$$\text{Viscerosomatic index (VSI, \%)} = \frac{\text{Weight of viscera (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{\text{Weight of liver (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Spleen somatic index (SSI, \%)} = \frac{\text{Weight of spleen (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Visceral fat-somatic index (VFSI, \%)} = \frac{\text{Weight of viscera fat (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Apparent protein deposition (APD, g)} = \text{Protein content of fish at end of study (g)} \\ - \text{protein content of fish at beginning of study (g)}$$

$$\text{Apparent Net Protein utilization (ANPU)} = \frac{\text{APD per fish}}{\text{Protein consumed}}$$

Nonspecific immune parameters. — Hematocrit (Hk, %) was calculated as a percentage of the red blood cells as:

$$\text{Hk (\%)} = \frac{\text{Length of packed red blood cells (mm)}}{\text{Length of whole blood (mm)}} \times 100$$

Hemoglobin (g/dL) was determined by the cyanmethemoglobin method (Houston 1990).

Eight μL of whole blood was added to 1 mL of transformation solution made by dissolving 1.0 g of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), 0.1 g of potassium cyanide (KCN), and 1.95 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in 1 L deionized water.

Cyanmethemoglobin was used as a standard and readings were taken at 540nm in a 96 well plate (200 μL) using a BioTek Epoch plate reader (Winooski, VT). The mean corpuscular hemoglobin content (MCHC) was calculated as the hemoglobin (g/dL) content divided by hematocrit (%).

Statistical analysis. —All data were analyzed by One-Way Analysis of Variance (ANOVA) using Minitab 17 (Minitab Inc. State College, PA). Percentage data from relative growth, VSI, HSI, VFSI, Hk and whole body protein content were \log_{10} transformed; SGR and SSI data were square root transformed before analysis. Tukey's HSD test was used to identify differences among treatment means. Means were considered different at $p < 0.05$.

Results

Replacement of FM by ACCM in diets of RBT resulted in differences in growth performance (Table 5.3) and VFSI (Table 5.4). Replacement of more than 25% of FM in RBT diets reduced ($p < 0.01$) weight gain. However, replacement of 25, 50 and 75% of FM provided a similar weight gain. Relative growth ($p < 0.01$) and SGR ($p < 0.01$) of RBT fed the reference diet were similar to those of RBT fed diets with 25 and 75% of FM replaced but higher than those of RBT fed the diet with 50% of FM replaced. The relative growth and SGR of RBT fed diets with FM replaced were similar. K values decreased ($p < 0.01$) with FM replacement. No mortality occurred during the experiment. Consumption of the reference diet was similar to consumption of the diet with 75% of FM replaced but better ($p < 0.01$) than the consumption of diets with 25% and 50% of FM replaced. The consumption of all diets with FM replacements were similar. The FCR of the diet with 25% of FM replaced was similar to that of the diet with 50% of FM replaced but better ($p < 0.01$) than that of the reference diet and the diet with 75% of FM replaced. The reference diet and the diets with 50 and 75% of FM replaced had a similar FCR. The amount of protein consumed ($p < 0.01$) associated with feed consumption. Fish fed the diet with 25% of FM replaced had a better ($p < 0.01$) PER than the PER of other diets

including the reference diet. The APD ($p=0.49$), whole-body protein ($p=0.09$), ash ($P=0.46$) and moisture ($P=0.09$) contents were similar among fish fed diets with FM replacements. However, the ANPU of the diet with 75% of FM replaced was lower ($p=0.01$) than the ANPU of diets with 25 and 50% of FM replaced. The ANPU of diets with 25 and 50% of FM replaced were similar. VSI ($p=0.39$), SSI ($p=0.41$) and HSI ($P=0.36$) were similar among dietary treatments. However, the trend in VFSI ($p=0.01$) associated with the trend in feed consumption. Hk ($P=0.81$), Hb ($P=0.13$) and MCHC ($P=0.16$) were unaffected by any level of FM replacement in RBT diets.

Discussion

Weight gain of the reference diet was high because of the higher feed consumption and protein intake. The lower consumption of the diet with 25% of FM replaced was compensated for by low FCR and high PER, which resulted in similar weight gain between fish fed the diet with 25% of FM replaced and the reference diet. All diets met the requirement for seven essential amino acids but the requirement for lysine and threonine were not fully met (Table 5.2). The requirement for arginine was only met by the reference diet and the diet with 25% of FM replaced. To meet the requirement for arginine, lysine and threonine based on the analyzed concentrations in the diets, all diets would have been supplemented with about 0.3% of arginine, 2.3% of lysine instead of 1.0% and 0.7% of threonine. Alternatively, to improve the lysine contents of the diets, the amount of wheat gluten would have been increased with reduction of FM and corn gluten. This is because the lysine content of wheat gluten (4.9%) is similar to that of menhaden fish meal (4.81%, NRC 2011) and higher than that of corn gluten (1.9%).

Also, ingredients such as blood meal that are high in lysine (7.1-8.2%), could be included in the diets.

Most available data on performance of meals containing GLS in fish diets were from the use of rapeseed (RSM) (Burel et al. 2000, 2001) or its cultivar (canola) which contains lower GLS contents (Thiessen et al. 2003, 2004, Shafaeipour et al. 2008; Slawski et al. 2013). However, recent studies have also evaluated camelina (Bullerwell et al. 2016; Ye et al. 2016) and carinata (Anderson et al. 2018) meals. The reference diets used in the studies above contained FM ranging from 32.1 to 56% of diet and total animal meals ranged from 37.1 to 62%.

Burel et al. (2000) replaced 34-60% of FM in a reference diet containing 53% FM, with 30-50% of different processed RSMs and all FM replacements resulted in reduced weight gain. However, Burel et al. (2001) replaced 9-49% of FM with 10 to 50% of different processed RSM in a reference diet containing 48% FM and did not observe reductions in growth up to inclusion levels of 30% of the diets with RSM. RSM dietary treatments that resulted in similar growth had between 31.5 and 43.5% FM. Reductions in growth observed in both studies by Burel et al. (2000 and 20001) were attributed to the presence of GLS in diets, whose breakdown products resulted in changes in thyroxine production, in addition to negative effects of other antinutrients. From Burel et al. (2001), it should be noted that inclusion of 30% of the two types of processed RSMs resulted in diets with 4.1 and 7.3 μmoles of GLS/g of feed. Additionally, iodine supplementation to these diets improved fish growth as opposed to the study in Burel et al. 2000, where a 30% RSM diet containing 1.4 μmoles of GLS/g of feed caused reduced fish growth without iodine supplementation. Thus, iodine supplementation mitigates the negative

effects of GLS breakdown products on fish growth. The initial fish size (20g) in Burel et al. (2000) was similar to the initial fish size in the current study. However, weight gains (45.2-70.6g) of RBT fed 53% FM after 63 days in Burel et al. (2000) were lower than weight gains (76.7-85.5g) of all RBT after 56 days in the current study, resulting in lower relative growth rates per day ($\leq 6.1\%$) compared to relative growth rates (6.6-7.8%) observed in the current study. Starting with a size of 22g, fish in Burel et al. (2001) gained lower weights (44.3 to 64.6g) after 58 days of feeding diets that contained more FM (24.5 to 48%) than what was contained in the current study. Relative growth rates per day ($\leq 5.1\%$) of fish in Burel et al. (2001), are lower than what was observed (6.6-7.8%) in the current study. It should be noted that weight gains by fish fed the reference diets in the above two studies were lower than the weight gain of fish fed the diet with 25% of FM replaced in the current study.

Thiessen et al. (2003) did not observe a difference in growth when canola fines (20%) were incorporated in the diet of RBT at a similar FM content (33%) as the control diet. Weight gain (136.6g) in the study (84 days) by Thiessen et al. (2003) was much higher than what was observed in the current study because the initial fish size (36g) and study duration were higher than comparative measurements in the current study. Relative growth rate per day (4.5%) of fish in Thiessen et al. (2003) was lower than what were observed in the current study. In the first experiment of Thiessen et al. (2004), they replaced 50 and 75% of the protein supplied by 62.5% of FM in the reference diet of RBT with canola protein concentrate (CPC), with and without feed attractants; and also with water washed CPC without attractants. There were no observed differences in growth and the weight gained (72.7-84.7g) after 63 days, similar to weight gain in the

current study. However, the study duration and initial fish size (28g) were higher than comparative measurements in the current study. Relative growth rates per day (4.1-4.8%) of fish in Thiessen et al. (2004) were lower what were observed in the current study.

Shafaeipour et al. (2008) did not observe any reduction in growth when solvent extracted canola meal replaced up to 58% of FM in the reference diet of RBT containing 52% FM. Their initial fish size (~4.2g) was smaller than the initial fish size in the current study, resulting in less weight gain (36.9-42.4g). However, their diets contained higher FM (22-58%) than the current study diets in addition to blood meal (10%) and the study duration (112 days) was twice the duration of the current study. Therefore, lower weight gain of RBT in their study could be due to a lower growth potential of the fish as shown by the low SGRs (1.9-2) for that size of fish. The relative growth rate per day of 9% is higher than what were observed in the current study but it is low for fish grown from 4.2g for 112 days.

Slawski et al. (2013) replaced 25, 50, 75 and 100% of FM with canola protein isolate (CPI) in the reference diet of RBT containing 32.5% FM. Fish fed the diet with CPI replacing 75% of FM grew better than the reference diet in the 70-day trial. Statistically, growth was similar among other test diets but the reference diet ranked lowest for performance. The highest weight gain (76.2g) by RBT was comparable to weight gain by RBT fed diets with 25 and 50% of FM replaced in the current study yet the initial size of fish fed that diet was 31.7g and the diet contained 8.1% FM and 15% blood meal. Relative growth rate per day (3.4%) was lower than relative growth rates per day for RBT in the current study.

Bullerwell et al. (2016) replaced 6.0, 11.3, 17.3 and 20.0% of FM in a reference diet containing 33.5% FM, with 5, 10, 15 and 20% of pre-press solvent extracted camelina meal (SECM), respectively, in RBT diets. Replacements of more than 11.3% FM with SECM reduced growth. Starting with a similar fish size (~19.5g), if growth of fish fed the reference diet and the diet in which 25% of FM was replaced with ACCM in the current study are compared for the same growth duration (56 days), weight gains in the above study (65.8-68.1g) were lower, resulting in lower relative growth rate per day (6.0-6.2%). In another experiment, Bullerwell et al. (2016) did not observe differences in growth of RBT fed diets with up to 20% of ground camelina seed meal or high oil residue camelina meal replacing up to 17% of FM in the reference diet containing 33% FM. The test diets in the second experiment contained $\geq 27.4\%$ of FM, 5% of feather meal and 5% of poultry by-product meal. Starting with a similar size (~20.4-22g), if growth of fish in the above study are compared to fish growth in the current study for the same growth duration (56 days), weight gains of all fish in the second experiment (<50g) were lower, resulting in lower relative growth rate per day (<4.4%).

Ye et al. (2013) replaced 5.6, 11.5, 17.4 and 23.4% of FM in a reference diet containing 32.1% FM with 5, 10, 15 and 20% of SECM, respectively, and did not observe any difference in growth. All diets used by Ye et al. (2013) contained 5% poultry by-product meal. Though the initial fish size (~8.4g) in the study by Ye et al. (2013) was lower than the initial fish size in the current study, weight gains (34.2-40.8g) after 112 days (2X the trial period of the current study) were much lower than what would have been obtained if the fish were to start at the same size as in Ye et al. (2013) but

maintained the performance of the current study fish. Relative growth rates per day (3.6-4.3%) of RBT in the study by Ye et al. (2013) were low for that size of fish.

Anderson et al. (2018) replaced 28.6% of FM with soaked and heated carinata or camelina meal, included at 15% of diets of RBT fingerlings. After 111 days of feeding, they did not observe reductions in growth of RBT fed carinata compared to the control. However, camelina reduced weight gain. A high relative growth rate per day of 28.2-34.1% observed in the study by Anderson et al. (2018) was because of the very small initial size (2.3-2.7g) of RBT used.

It is not clear if the strain of RBT in the current study had a better growth potential than those of RBT used in most of the above studies, resulting in better performance. However, it is worth noting that more ACCM may have been tolerated because of higher FM or animal meal content in the diets as in the above studies.

The trend in relative growth and SGR did not fully associate with the trend in weight gain among dietary treatments. There was no difference in relative growth or SGR between the reference diet and diet with 75% of FM replaced because the variability in growth data was slightly increased when growth was expressed as a percentage of the initial weights. SGRs in the current study were better than the SGRs in the studies by Burel et al. 2000, 2001; Slawski et al. (2013) and Ye et al. (2013). K values decreased with increased ACCM due to a combined effect of lower feed intake and protein efficiency.

There was no lethal effect of ACCM on RBT in the current study because all fish survived throughout the study. Shafaeipour et al. (2008) and Slawski et al. (2013) also reported no effect of including canola meal or protein isolate on RBT survival. Studies by

Burel et al. (2000, 2001) and Thiessen et al. (2003, 2004) on different processed RSM/canola meals; and Ye et al. (2016) and Bullerwell et al. (2015) on SECM, did not mention any lethal effects of including canola or camelina meals in diets of RBT, which may suggest that the two seed meals did not negatively affect fish survival.

Feed consumption was expected to decrease with reduced FM in the diets as observed in diets in which 25 and 50% of FM was replaced. However, consumption of the reference diet was similar to the diet with the highest FM replacement, which shows that ACCM alone may not be responsible for reduced feed consumption in lower FM replacement diets. ACCM did not affect feed consumption when included in the diets of Hybrid Striped Bass up to 30% (Table 6.3). Burel et al. (2000, 2001), Thiessen et al. (2003, 2004), Slawski et al. (2013), Ye et al. (2016) and Bullerwell et al. (2015) also reported no negative effects of replacing FM with RSM/canola or camelina seed meals on feed consumption. Burel et al. (2000) observed that fish adapted to the diets after 3 weeks, resulting in similar or better consumption than the reference diet. Shafaeipour et al. (2008) observed reduced feed consumption with FM replacement with canola meal but the trend was inconsistent with FM replacement levels because higher replacements resulted in better consumption, similar to the current study.

The FCRs improved with less feed intake but differences among treatments were very small and likely not biologically significant. Low FCRs of ≤ 1.2 were reported by Thiessen et al. (2003, 2004), Slawski et al. (2013), Bullerwell et al. (2016) and Ye et al. (2016) in RBT for all replacements of FM by canola or camelina seed meals. The FCRs (1.01-1.47) reported by Burel et al. (2000) worsened with replacement of FM by RSM. However, it is only the highest replacement of FM in Burel et al. (2001) that increased

the FCR (1.64) beyond 1.2. Most FM replacements by SECM in Shafaeipour et al. (2008), resulted in FCRs ≤ 1.2 except for the diet with the second highest SECM content, whose FCR (1.5) was different from other FCRs (1.0-1.2). Gomez-Requeni et al. (2004) also observed lower FCRs with less feed consumption in gilthead Seabream *Sparus aurata* fed diets where FM was replaced with a blend of plant meals consisting of corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin, as observed in the current study. In both studies, the reduction in feed consumption is attributed to FM replacement by plant meals in the diets.

Protein consumed in the current study associated with feed consumed because the protein contents of the diets were similar. Protein efficiency was not well associated with FCR because fish fed the diet with 50% of FM replaced had both low weight gain and consumed less protein. The PERs observed in the current study are higher than the PERs observed in the studies by Thiessen et al. (2003, 2004) but comparable to the PERs observed in the studies by Slawski et al. (2013) and Shafaeipour et al. (2008). However, all the above studies used more FM or animal meals in their diets.

There appeared to be a decreasing trend in the ANPU with increasing ACCM in the diets but fish fed the diet with 75% of FM replaced had a lower ANPU yet protein intake was higher than those of fish fed diets with 25 and 50% of FM replaced. Diets with 50 and 75% of FM replaced also did not meet the requirements for arginine in addition to lysine and threonine and this may also explain the decreasing trend in ANPU with increasing ACCM in the diets.

The visceral fat content of RBT did not uniformly increase with increasing ACCM in the diets and may not be directly explained by the composition of ACCM. The

soluble starch in ACCM is also expected to be low as soluble components of the meal are washed out in the wash step involved in processing ACCM. The other plant based ingredients that contain starch and varied in the diets were wheat gluten and whole cleaned wheat. However, wheat gluten production involves isolation of starch resulting in a meal that is low in starch (Day et al. 2006). Whole cleaned wheat decreased with increasing ACCM in formulations and although this may partly explain the high viscera fat in RBT fed the reference diet, it does not explain why fish fed the diet with 25% of FM replaced had the lowest viscera fat content yet that diet had the second highest content of whole cleaned wheat and lowest content of ACCM. Therefore, since the nitrogen free extracts (NFE) in all diets were similar, viscera fat content associated with the amount of feed and thus consumed NFE.

Conclusion

Overall growth of RBT in the current study was higher or comparable to growth of RBT of similar size in most studies, although the diets used contained low FM ($\leq 20\%$)/animal meal. However, any replacements of FM by ACCM beyond 25%, resulted in reduced fish growth. Reduced fish growth may partly be attributed to reduced feed consumption. However, protein retention reduced with FM replacement by ACCM. It is probable that RBT would have tolerated more ACCM in the diets if the total animal contents were higher in the test diets. Therefore, future studies should test the performance of ACCM in RBT diets at reduced FM but with similar or higher total animal contents than what were used in the current study. Increased animal contents in the diets will improve diet amino acid composition, palatability, digestibility and protein utilization; resulting in higher fish growth.

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Table 5.1. Formulation (g/100g, dry basis) of diets used to test the performance of processed carinata meals in Rainbow Trout^a.

Ingredient	Reference 20% FM ^a	Carinata		
		5% ACCM ^a	10% ACCM ^a	15% ACCM ^a
Fish meal (special select) ^b	20.0000	15.0000	10.0000	5.0000
ACCM ^c	0.0000	5.0000	10.0000	15.0000
Yellow corn gluten ^d	19.5000	21.0000	22.3000	24.0000
Wheat gluten ^e	10.0000	10.0000	10.0000	10.0000
Whole Cleaned Wheat ^f	26.6475	24.3475	22.4975	20.2375
Brewers yeast ^g	0.2000	0.2000	0.2000	0.2000
CMC ^h	2.1000	1.4000	0.7000	0.0000
Vitamin premix ⁱ	1.0000	1.0000	1.0000	1.0000
Mineral premix ⁱ	0.0500	1.0000	1.0000	0.0500
Stay C ^j	0.2000	0.2000	0.2000	0.2000
Choline (60%) Chloride ^k	0.3000	0.3000	0.3000	0.3000
Arginine ^l	0.5200	0.5200	0.5200	0.5200
Lysine ^m	1.0000	1.0000	1.0000	1.0000
Methionine ⁿ	0.5000	0.5000	0.5000	0.5000
Cysteine ^l	0.1000	0.1000	0.1000	0.1000
Taurine ^o	0.5000	0.5000	0.5000	0.5000
Betaine ^l	0.5000	0.5000	0.5000	0.5000
Sodium chloride ^p	0.5000	0.5000	1.0000	1.6000
Potassium chloride ^q	0.8000	0.8000	0.8000	1.5000
Monocalcium phosphate ^r	0.5000	0.7500	1.3000	2.0000
Calcium propionate ^s	0.0025	0.0025	0.0025	0.0025
Sodium Bicarbonate ^f	0.2500	0.2500	0.2500	0.2500
Lecithin ^t	3.0000	3.0000	3.0000	3.0000
Menhaden, VA prime gold ^b	11.8300	12.1300	12.3300	12.5400
Proximate composition				
Crude protein (%) ^u	44.3	44.1	43.6	44.3
Crude lipid (%) ^v	16.9	17.0	16.9	16.9
Crude fiber (%) ^v	3.2	3.2	3.2	3.2
Ash (%) ^v	9.7	9.6	9.6	9.6
NFE (%) ^v	25.9	26.1	26.7	26.0
Gross energy (GE, MJ/Kg) ^v	22.1	22.0	20.0	20.0
Protein:Energy (g/MJ) ^v	19.1	19.1	19.1	19.1

^aDiets formulated to contain 42% crude protein and 14% crude lipid. Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of fish meal (FM) in the reference diet; ^bOmega proteins, Houston, TX; ^cSouth Dakota State University, Brookings, SD; ^dKent Nutrition Group, Muscatine, Iowa; ^eManildra Milling Corporation, Leawood, KS; ^fAg First Farmers Co-op, Brookings, SD; ^gDiamond V Mills Inc., Cedar Rapids, Iowa; ^hAkzo Nobel Functional Chemicals BV, Amersfoort, Netherlands; ⁱNutra Blend LLC, Neosho, MO; ^jDSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China; ^kBiochem Corporation, New Hampton, NY; ^lPure Bulk, Rosenberg, OR; ^mAnjinomoto Animal Nutrition group, Chicago, IL.; ⁿAdisseo Blue Star Company, North Point Parkway, GA; ^oJiangyin, Huachang Food Additives, Jiangyin Jiangsu, China.; ^pCompass Minerals America Inc., Overland Park, KS; ^qPhibro Animal Health Corporation, Teaneck, NJ; ^rPCS Sales, Northbrook, IL; ^sAcros Organics, Morris Plains, NJ; ^tSolae LLC, St. Louis, MO; ^uAnalyzed; ^vCalculated.

Table 5.2. Analyzed amino acid composition (g/100g, dry basis) of diets used to test the performance of processed carinata meals in Rainbow Trout^a.

Amino acid	Reference 20% FM ^a	Carinata			Requirement ^b
		5% ACCM ^a	10% ACCM ^a	15% ACCM ^a	
Essential					
Arginine	1.57	1.48	1.22	1.22	1.5
Histidine	0.83	0.79	0.71	0.80	0.8
Isoleucine	3.93	4.06	3.62	4.26	1.1
Leucine	1.95	1.99	1.80	2.07	1.5
Lysine	1.68	1.84	1.75	1.17	2.4
Methionine ^c	1.53	1.69	1.64	1.33	0.7
Phenylalanine	1.95	1.99	1.80	2.07	0.9
Threonine	1.19	1.17	1.05	1.18	1.8
Tryptophan	ND	ND	ND	ND	0.3
Valine	1.82	1.82	1.59	1.94	1.2
Conditionally essential					
Cysteine ^d	1.41	1.32	1.32	1.08	0.4
Tyrosine	1.55	1.57	1.39	1.44	-
Non-essential					
Alanine	2.08	2.09	1.79	2.06	-
Aspartate	1.94	2.15	1.98	1.59	-
Glutamate	8.82	9.02	8.67	8.39	-
Glycine	1.58	1.49	1.25	1.28	-
Proline	3.10	3.16	2.79	3.18	-
Serine	1.65	1.68	1.49	1.68	-

^aDiets formulated to contain 42% crude protein and 14% crude lipid. Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of fish meal (FM) in the reference diet; ND = Not detected, ^bNRC (2011).

Cysteine calculated as a difference in methionine and combined methionine+cysteine requirements;

^cMethionine sulfoxide; ^dCysteic acid.

Table 5.3. Effects of replacing fish meal with processed carinata meals on performance variables of Rainbow Trout^a.

Variable	Reference 20% FM ^a	Carinata			P-value
		5% ACCM ^a	10% ACCM ^a	15% ACCM ^a	
Initial weight/fish (g)	19.69±0.21	20.03±0.19	20.00±0.17	19.79±0.18	0.518
Final weight/fish (g)	105.18±1.64 ^a	100.08±3.23 ^{ab}	93.42±2.15 ^b	96.49±1.02 ^b	0.004
Weight gain/fish (g)	85.49±1.60 ^a	80.05±3.17 ^{ab}	73.4±2.17 ^b	76.7±1.16 ^b	0.004
Weight gain/day (g)	1.53±0.03 ^a	1.43±0.06 ^{ab}	1.31±0.04 ^b	1.37±0.02 ^b	0.004
Relative growth (%)	434.4±8.66 ^a	399.6±15.31 ^{ab}	367.3±11.81 ^b	387.9±8.77 ^{ab}	0.005
SGR (%) ^b	2.99±0.03 ^a	2.87±0.06 ^{ab}	2.75±0.05 ^b	2.83±0.03 ^{ab}	0.005
Fulton's K	1.34±0.02 ^a	1.32±0.02 ^{ab}	1.26±0.02 ^b	1.18±0.01 ^c	<0.001
Survival (%)	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	-
Feed consumed (g)	1870±40.29 ^a	1663±58.59 ^b	1591±38.18 ^b	1704±34.57 ^{ab}	0.001
FCR ^c	1.09±0.01 ^a	1.04±0.01 ^b	1.08±0.01 ^{ab}	1.1±0.01 ^a	0.001
Protein consumed (g)	828.2±17.8 ^a	733.7±25.9 ^b	694.5±16.7 ^b	754.7±15.3 ^{ab}	<0.001
Wet biomass gained (g)	1720.1±31.4 ^a	1601.0±63.4 ^{ab}	1543.5±45.8 ^b	1477.8±26.0 ^b	0.004
PER ^d	2.19±0.01 ^b	2.29±0.03 ^a	2.21±0.02 ^b	2.16±0.02 ^b	0.001
APD/fish (g) ^e	ND ^g	14.27±0.83	13.87±0.75	13.07±0.41	0.493
ANPU/fish ^f	ND ^g	0.39±0.01 ^a	0.38±0.01 ^a	0.34±0.00 ^b	0.006
Whole-body protein (% wet)	ND ^g	17.26±0.84	16.90±0.75	16.03±0.40	0.480
Whole-body ash (% wet)	ND ^g	2.59±0.16	2.53±0.03	2.40±0.06	0.464
Whole-body moisture (%)	ND ^g	66.52±0.79	64.51±0.11	65.46±0.41	0.087

^aDiets formulated to contain 42% crude protein and 14% crude lipid. Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of fish meal (FM) in the reference diet. Values are means±SE; ^bSpecific growth rate; ^cFeed conversion ratio; ^dProtein efficiency ratio; ^eApparent protein deposition; ^fApparent net protein utilization; ^gNot determined because sorted samples for the reference diet were mistakenly discarded before analysis when cleaning out the freezer.

Table 5.4. Effects of replacing fish meal with processed carinata meals on organosomatic indices and blood parameters of Rainbow Trout^a.

Variable	Reference 20% FM ^a	Carinata			P-value
		5% ACCM ^a	10% ACCM ^a	15% ACCM ^a	
VSI (%) ^b	15.22±0.52	14.27±0.16	14.03±0.47	14.76±0.65	0.386
SSI (%) ^c	0.11±0.01	0.10±0.01	0.11±0.01	0.10±0.01	0.411
HSI (%) ^d	1.55±0.04	1.51±0.06	1.44±0.05	1.57±0.07	0.356
VFSI (%) ^e	3.60±0.11 ^a	2.70±0.13 ^b	3.42±0.14 ^a	3.03±0.20 ^{ab}	0.009
HK (%) ^f	43.88±1.09	43.13±0.90	42.25±1.36	42.57±2.13	0.811
Hb (g/dL) ^g	8.91±0.50	9.47±0.46	8.69±0.66	7.73±0.34	0.125
MCHC (g/dL) ^h	20.35±1.14	21.99±1.00	20.49±1.22	18.11±1.22	0.160

^aDiets formulated to contain 42% crude protein and 14% crude lipid. Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of fish meal (FM) in the reference diet. Values are means±SE; ^bViscerosomatic index; ^cSpleen somatic index; ^dHepatosomatic index; ^eVisceral fat somatic index; ^fHematocrit; ^gHemoglobin; ^hMean corpuscular hemoglobin content.

CHAPTER 6

PERFORMANCE OF HYBRID STRIPED BASS *Morone chrysops* ♀ X *M. saxatilis* ♂ FED DIETS CONTAINING CARINATA *Brassica carinata* SEED MEALS

Abstract

Carinata *Brassica carinata* is an oilseed crop undergoing genetic modification to increase the seed oil and erucic acid content for jet fuel production. The solvent extracted seed meal may be used in animal diets because of its high protein (>40%) content. However, its utilization in fish diets is limited by high crude fiber (>9%), glucosinolates and sinapine. We processed solvent extracted carinata meal by aerobic conversion (AC) followed by a single wash (ACCM), or a double wash (WCM) without AC. Four diets were formulated to contain ~42% protein and 12% lipid, including a fish meal (FM) reference diet. All diets contained 10% FM and 10% chicken by-product meal and test diets contained 10 or 30% ACCM, or 30% WCM.

A 106-day trial was carried out in a recirculating aquaculture system to compare the growth performance of Hybrid Striped Bass (HSB) fed reference and test diets. Survival (>99%) was similar among treatments. The HSB fed 30% WCM had a similar weight gain to HSB fed the FM reference diet and 30% ACCM but better than HSB fed 10% ACCM ($p=0.01$). Relative growth ($p=0.02$) and specific growth rate ($p<0.01$) followed the same trend as weight gain. Fish fed 30% WCM and 30% ACCM had a similar feed consumption to that of fish fed 10% ACCM but better than fish fed the FM reference diet ($p<0.01$). Fish fed the FM reference diet had a similar consumption to those fed 10% ACCM diets. Feed conversion ratio (FCR) of fish fed 30% WCM was similar to that of fish fed the reference diet but better than that of fish fed 10% and 30%

ACCM ($p < 0.01$). Protein efficiency ratio (PER) of fish fed 30% WCM was similar to that resulting from the reference but better than 10% ACCM and 30% ACCM diets ($p < 0.01$). The ACCM diets had a lower PER than the reference diet. Protein deposition was highest in fish fed the highest carinata meals ($p = 0.02$). The trend in apparent net protein utilization ($p = 0.01$) was similar to that of FCR. Whole body protein significantly increased with increasing carinata in the diets ($p = 0.01$). No dietary effect was observed for whole-body ash or moisture contents. The viscera-somatic, spleen-somatic and viscera fat indices were similar among treatments. However, HSB fed 30% WCM had smaller livers and higher condition factors than fish fed other treatment diets. Hematocrit ($p = 0.01$) was high in fish fed ACCM but the increase did not associate with the amount of ACCM in the diet. Hemoglobin (Hb, $p < 0.01$) contents of HSB were increased by ACCM but not WCM. There was no dietary treatment effect on lysozyme or ACH₅₀.

Our results show that processed carinata seed meal is a viable protein ingredient for fish diets. In particular, double washing improved feed conversion and protein efficiency more than aerobic conversion with a single wash. Carinata seeds are high in iron and this may account for the differences observed Hk and Hb responses among dietary treatments, given that the extra wash step in WCM likely reduced the iron content of the meal.

Introduction

Carnivorous fish utilize mostly protein and lipid in nature for nutrients and energy (De Silva and Anderson 1994) making them less efficient at utilizing high levels of dietary carbohydrates in artificial feeds under culture conditions (Wilson 1994; Moon 2001). The carbohydrate (starch) content of carnivorous fish feeds is normally less than

20% (Wilson 1994). The digestible proteins reported for most carnivorous fish are above 36% (NRC 2011). However, such low digestible protein requires high lipids in diets to meet both energy and essential fatty acid requirements so that protein is spared for growth (Lee and Lee 2005). Carnivorous fishes require highly unsaturated fatty acids (HUFAs) such as eicosapentanoic, docosahexaenoic and arachidonic acids, as essential fatty acids (Tocher 2003). The nutritional requirements of carnivorous fish render fish meal (FM) and oil ideal for use in their diets (De Silva and Anderson 1994). However, higher FM demand due to increased aquaculture production and uses in other animal feeds has increased FM prices (FAO 2016). This need for high protein has stimulated increased research on use of alternative protein sources such as oilseeds and cereal grains in fish feeds.

Plant meals are generally lower in protein, essential amino acids and minerals than most animal feedstuffs; and do not contain HUFAs (NRC 2011). Therefore, high replacements of FM with plant meals in carnivorous fish feeds would require essential amino acid and mineral supplementation, and use of oils high in HUFAs. Plant meals also contain antinutritional factors (ANFs, Francis et al. 2001) that reduce diet palatability and nutrient digestibility (Krogdahl et al. 2010).

Genetic modification of Carinata *Brassica carinata* oilseed is intended to increase its oil and erucic acid contents (Taylor et al. 2010), which will increase its production for fuel oil and thus availability of de-oiled carinata meals. The protein content of carinata seeds ranges from 25.9 to 30.5% (Pan et al. 2012) and the lipid content is about 41% (Xin et al. 2013), resulting in a de-oiled meal that is high in protein (>40%). De-oiled carinata meal contains some taurine and the essential amino acid profile (Table 3.3) is inferior to

that of FM (NRC 2011) but comparable to that of de-oiled soybean meal with hulls (NRC 2011). Carinata oil contains 13.7 to 18.9% linoleic acid and 10.2-16.0% linolenic acid (Warwick et al. 2006).

Carinata meal also contains ANFs such as glucosinolates (GLS), phytates, (Rakow and Getinet 1998; Pedroche et al. 2004), tannins (Xin et al. 2014), erucic acid (Warwick et al. 2006) and sinapine (Mailer et al. 2008) in addition to non-starch polysaccharides (NSPs). Genetic modification of carinata seeds is likely to alter the concentration of the aforementioned ANFs that are inherent. These ANFs have to be removed or reduced to levels tolerated by fish in order to provide an acceptable ingredient for feed applications. Some ingredient processing methods such as soaking and extrusion can lower the concentration of most ANFs in plant meals but not NSPs. Although some components of NSPs can stimulate nonspecific immune responses in fish (Sinha et al. 2011), NSPs' negative effects on nutrient digestibility (Simon et al. 1996) outweigh immune benefits. NSPs in plant meals can be reduced by use of exogenous enzymes (Adeola and Cowieson 2011) and /or microbial fermentation (Pandey et al. 2000). The increase in protein due to microbial fermentation is due to increased single cell microbial mass through sugar utilization; and removal of fiber and soluble seed components, resulting in a higher concentration of the residual protein. The increase in protein in fermented carinata meal would allow less carinata meal to replace fish meal on an equal protein basis.

Hybrid Striped Bass (HSB) *Morone chrysops* x *M. saxatilis* is produced from a cross of female White Bass and male Striped Bass, which enables HSB to acquire better culture characteristics from both parents such as faster growth rates, better survival, disease resistance and tolerance to variable water quality (Morris et al. 1999). HSB is

among the top four major food fish produced in the US (USDA-NASS 2012). The optimal dietary protein content for growth of HSB was determined to be about 40% (Brown et al. 1992) and therefore current commercial HSB diets contain $\geq 40\%$ protein.

The objective of this study was to determine if up to 30% ACCM or 30% WCM would be tolerated in HSB diets at a similar animal protein content without affecting fish growth. The results of this study demonstrate how processing improves the nutritional value of carinata meal.

Materials and Methods

Source of and preparation of feed ingredients. —Menhaden fish meal (Special Select) and oil (Omega Prime) were obtained from the Omega Protein Corporation (Houston, TX). Hexane extracted (HE) carinata meal was contributed by Agrisoma Biosciences Inc., Quebec, Canada. A portion of the HE carinata meal was aerobically converted (AC) using fungi spp. Followed by a single wash, to produce ACCM and the other portion was doubled washed to produce washed carinata meal (WCM) (W. Gibbons, personal communication, South Dakota State University). Modified soybean meal was contributed by Prairie Aquatech LLC (Brookings, SD). Chicken by-product meal was obtained from Tyson Foods Inc. (Springdale, AR). Corn protein concentrate (Empyreal75) was obtained from Cargill Inc. (Blair, NE). Wheat midds were obtained from Nutra Blend LLC (Neosho, MO). Corn and whole clean wheat were obtained from Ag First Farmers Co-op (Brookings, SD). Wheat gluten was obtained from the Manildra Milling Corporation (Leawood, KS). Carboxymethyl cellulose was obtained from Akzo Nobel Functional Chemicals BV (Amersfoort, Netherlands). Vitamin and mineral premixes were specialty blends for fish diets. Stay C was obtained from DSM Jiangshan

Pharmaceutical Co. Ltd (Jingjiang, Jiangsu, China). Choline (60%) chloride was obtained from Biochem Corporation (New Hampton, NY). Lysine and tryptophan were obtained from the Anjinomoto Animal Nutrition Group (Chicago, IL). Methionine was obtained from the Adisseo Blue Star Company (North Point Parkway, GA). Monocalcium phosphate was obtained from PCS Sales (Northbrook, IL) and soybean oil was provided by South Dakota Soybean Processors (Volga, SD).

Composition of feed ingredients. —Dry matter (AOAC 2006, method 934.01), crude protein (AOAC 2006, method 972.43), crude lipid (AOAC 2006, method 2003.06), crude fiber (AOAC 2006, method 962.09), and ash (AOAC 2006, method 942.05) contents were analyzed for processed carinata meals, as well as; moisture and protein contents of other ingredients prior to formulating diets. GLS were analyzed with a Shimadzu (Columbia, MD) HPLC system using a method developed by Betz and Fox (1994) with slight modifications.

Diet composition and manufacture. —Four isonitrogenous (~42% crude protein) and isocaloric diets (~4,795 Kcal/kg) were formulated for HSB (Table 6.1). Diets were formulated to meet known essential amino acid requirements (Table 6.2) of HSB based on ingredient amino acid compositions, and contained 20% animal meal as 10% fish meal and 10% chicken by-product meal, and ~12% crude lipid with 8% as fish oil. The reference diet contained no carinata meal and the test diets contained 10 and 30% ACCM or 30% WCM (Table 6.1). All dry ingredients were ground to particles less than 0.8 mm in a Fitzpatrick comminutor mill (Elmhurst, IL) prior to blending all ingredients in a Leland 100DA70 double action food mixer (Fort Worth, TX). The blended ingredients were cook extruded using an Extru-Tech E325 single screw extruder (Sabetha, KS) to

provide 3mm pellets that were passed through a model HC-1210 drier (Colorado Mill Equipment LLC, Canon city, CO). Dry pellets were vacuum coated with the remaining oil in a Phlauer high performance mixer (A&J Mixing International Inc., Ontario, Canada), then bagged and frozen at -20°C , pending use.

Fish culture and sampling. —This study was conducted under South Dakota State University IACUC protocol # 15-064A. Twenty randomly selected fish ($19.2 \pm 0.08\text{g}$) were stocked in each tank (106L) of a 32-tank, recirculating aquaculture system (RAS) and 10 fish of similar size were euthanized and frozen for composition analysis. Seven replicates of the reference diet and six replicates of each test diet were randomly assigned to the individual tanks. Temperature, pH and dissolved oxygen were monitored daily using a YSI Professional Plus meter (YSI, Yellow Springs, OH) and averaged (mean \pm SD) $25.6 \pm 0.7^{\circ}\text{C}$, 7.5 ± 0.5 and $9.0 \pm 1.1\text{ mg/L}$, respectively. Unionized ammonia and nitrite were monitored weekly using a Hach DR 2000 spectrophotometer (Hach, Loveland, CO) and averaged $0.01 \pm 0.005\text{ mg/L}$ and $0.15 \pm 0.07\text{ mg/L}$, respectively. Fish were fed twice a day to satiation and feed consumption per individual tank was recorded throughout the study. Tank weights were measured every three weeks to monitor growth.

At the end of the study (106 days), two fish from each tank were euthanized, weights and lengths were measured, and sampled for blood. One heparinized micro-capillary tube was filled with blood for each fish and centrifuged at 10,000 rpm to separate red blood cells (RBCs) for calculation of hematocrit (Hk) values. Additional blood was placed in a transformation solution for hemoglobin (Hb) analysis. The remaining blood was immediately placed on ice and later stored in the fridge (4°C) overnight. Blood was centrifuged at 1000 rpm in a chilled (4°C) centrifuge, serum was

collected, and pooled by tank and immediately frozen at -80°C , pending lysozyme and alternative complement activity analyses. The same fish were necropsied to collect viscera for isolation of the livers, spleens and viscera fat. The viscera, livers, spleens and viscera fat of each fish were weighed for calculation of organo-somatic indices. The remaining fish in each tank were also weighed and counted to determine total tank biomass and survival. Three other fish were euthanized, frozen and later homogenized to provide a homogenous sample per replicate. The 10 fish frozen at the beginning of the study were also homogenized. The homogenized fish samples were frozen and then freeze dried (Labconco Freezone 2.5 freeze-dryer, Kansas City, MI) for 72 hours. The dried fish samples were finely ground in a coffee grinder, mixed to ensure uniformity and analyzed for protein (AOAC 2006, method 972.43). Protein concentrations were used to calculate apparent protein deposition (APD) and apparent net protein utilization (ANPU).

Calculation of performance variables. —The growth performance variables were calculated using the following equations;

$$\text{Initial weight/fish (g)} = \frac{\text{Tank biomass at the beginning of study}}{\text{Number of fish in the tank}}$$

$$\text{Final weight/fish (g)} = \frac{\text{Tank biomass at end of study}}{\text{Number of fish in the tank}}$$

$$\text{Weight gain/fish (g)} = \text{Final weight} - \text{initial weight}$$

$$\text{Weight gain/day (g)} = \frac{\text{Weight gain}}{\text{Days of study}}$$

$$\text{Relative growth (\%)} = \frac{\text{Final weight} - \text{initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Specific growth rate (\%)} = 100 \times ((\ln(\text{final weight}) - \ln(\text{initial}))/\text{days of growth})$$

$$\text{Fulton's (1904) condition factor (K)} = \frac{\text{Weight (g)} \times 100}{\text{Length (cm)}^3}$$

where 100 is a factor required to bring the value of k near unity

$$\text{Survival (\%)} = \frac{\text{Number of fish at end of study}}{\text{Number of fish at start of study}} \times 100$$

Feed consumed (g) = Sum of feed fed from start to end of study.

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Dry feed fed for entire study(g)}}{\text{Wet biomass gained during study (g)}}$$

Protein consumed (g) = Feed consumed (g) x percentage of protein in feed

Wet biomass gained = Tank biomass at end of study (g) + biomass of mortalities during the study (g)

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Wet biomass gained during study(g)}}{\text{Protein consumed (g)}}$$

$$\text{Viscera-somatic index (VSI, \%)} = \frac{\text{Weight of viscera (g)} \times 100}{\text{Body weight (g)}}$$

$$\text{Hepato-somatic index (HSI, \%)} = \frac{\text{Weight of liver (g)} \times 100}{\text{Body weight (g)}}$$

$$\text{Spleen-somatic index (SSI, \%)} = \frac{\text{Weight of spleen (g)} \times 100}{\text{Body weight (g)}}$$

$$\text{Viscera fat-somatic index (VFSI, \%)} = \frac{\text{Weight of viscera fat (g)} \times 100}{\text{Body weight (g)}}$$

Apparent protein deposition (APD, g) = Protein content of fish at end of study (g)
– protein content of fish at beginning of study (g)

$$\text{Apparent Net Protein utilization (ANPU)} = \frac{\text{APD per fish}}{\text{Protein consumed}}$$

Nonspecific immune parameters. — Hematocrit (Hk, %) was calculated as a percentage of the red blood cells as;

$$\text{Hk (\%)} = \frac{\text{Length of packed red blood cells (mm)}}{\text{Length of whole blood (mm)}} \times 100$$

Hemoglobin (g/dL) was determined by the cyanmethemoglobin method (Houston 1990).

Eight uL of whole blood was added to 1 mL of transformation solution made by dissolving 1.0 g of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), 0.1 g of potassium cyanide (KCN), and 1.95 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in 1 L deionized water.

Cyanmethemoglobin was used as a standard and readings were done at 540nm in a 96 well plate (200 uL) using a Bio Tek Epoch plate reader (Winooski, VT). The mean corpuscular hemoglobin content (MCHC) was calculated as the hemoglobin (g/dL) content divided by hematocrit (%).

Serum lysozyme activity was quantified using a turbidimetric assay (Kim et al. 2006) by measuring reduction in optical density (OD) caused by lysis of *Micrococcus lysodeikticus*. 100 μL of serum was diluted four times in two-fold dilutions using 0.05M sodium phosphate buffer (SPB, pH 6.2) in 96 well plates and 100 μL of a suspension of 0.4 mg of *M. lysodeikticus* (Worthington Biochemical) per mL of PBS (pH 6.2) was added. 100 μL of SPB replaced serum as a negative control and 200 μL of SPB was used as a blank. The plates were incubated at 22°C and the O.D was measured at 570 nm with a Bio Tek Epoch plate reader (Winooski, VT) with readings at 0, 15 and 30 mins. A unit of lysozyme activity was defined as the amount of serum causing a decrease in the absorbance of 0.001min^{-1} .

Alternative complement activity (ACH50) was quantified as the amount of hemolysis of sheep RBCs as described by Welker et al. (2014). Sheep RBCs (10% packed volume in Alsever's solution; Lampire Biological, Pipersville, PA) were washed 4 times (300g at 4°C) with cold PBS+ (PBS containing 0.1% gelatin, 0.15mM CaCl_2 ,

and 0.5mM MgCl₂, pH 7.2) and then suspended in PBS+. The concentration of RBCs was determined using a hemacytometer and standardized to 5×10^7 RBCs mL⁻¹. From 100 µL of serum in the starting wells (As) of a 96 well plate, 50 µL of serum were transferred to 50 µL of PBS+, to start seven two-fold dilutions, resulting in serum volumes of 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.390625 µL as part of the 50 µL solutions in the eight wells (A-H). To each well containing the diluted serum, 200 µL of PBS+ was added, followed by the addition of 50 µL of washed sheep RBCs, resulting in a final volume of 300 µL. Positive (100% hemolysis) and negative (spontaneous hemolysis) controls were 250 µL of distilled water + 50 µL of RBCs and 250 µL of PBS+ + 50 µL of RBCs, respectively. Samples were incubated at 22°C for 1 hour but mixed every 15 min by gently tapping the sides of plates. The reaction was stopped by placing the plates on ice for 3 mins. Plates were centrifuged at 800g for 10 min at 4°C and 250µL of supernatant was transferred to a 96-well plate. The absorbance was measured at 410 nm using a Bio Tek Epoch plate reader (Winooski, VT). Alternative complement hemolytic (ACH) activity was expressed as ACH₅₀ units mL⁻¹, where one ACH₅₀ unit was equal to the volume of serum necessary to lyse 50% of sheep RBCs. The degree of hemolysis was estimated via the lysis curve for $Y / (1 - Y)$ against the volume of serum added. The Y value (percentage of hemolytic activity at each dilution relative to the positive and negative controls) was determined by the equation:

$$Y = 100 \times [\text{Abs}(A) - \text{Abs}(B)] / [\text{Abs}(C) - \text{Abs}(B)]$$

where, Abs (A) = absorbance of the HSB serum dilution; Abs (B) = absorbance of the negative control (spontaneous lysis); Abs (C) = absorbance of the positive control (100% RBC lysis).

Statistical analysis. —All data were analyzed using One-Way Analysis of Variance (ANOVA) using Minitab 17 (Minitab Inc. State College, PA). Percentage data from relative growth, VSI, HSI, VFSI, Hk and whole body protein content were \log_{10} transformed; and specific growth rate and SSI data were square root transformed before analysis. Tukey's HSD test was used to identify differences among treatment means. Treatment means were considered different at $P < 0.05$.

Results

Performance varied among dietary treatments fed different processed carinata meals (Table 6.3). Final weight ($p=0.01$), weight gain ($p=0.01$), relative growth ($p<0.01$) and SGR ($p=0.0016$) of fish fed the diet containing 30% WCM were similar to those of fish fed the reference diet and the diet containing 30% ACCM but higher than those of fish fed the diet containing 10% ACCM. Final weight, weight gain, relative growth and SGR of fish fed the reference diet were similar to those of fish fed diets containing 10 and 30% ACCM. The K value of fish fed the diet containing 30% WCM ($p<0.01$) was higher than those of fish fed other treatment diets. K values of fish fed the reference diet was similar to those of fish fed diets containing 10 and 30% ACCM. Fish survival ($p=0.72$) was unaffected by any dietary treatment.

Consumption of the diet containing 30% WCM was similar to that of diets containing 10 and 30% ACCM but higher ($p<0.01$) than that of the reference diet. The FCR of the diet containing 30% WCM was similar to that of the reference diet but higher ($p<0.01$) than those of diets containing 10 and 30% ACCM. The FCR of diets containing 10 and 30% ACCM were similar. The protein consumption of fish fed the diet containing 30% ACCM was similar to that of the diet containing 30% WCM but higher ($p<0.01$)

than those of fish fed the reference diet and the diet containing 10% ACCM. The protein consumption of fish fed the diet containing 30% WCM was similar to that of fish fed the diet containing 10% ACCM but higher than that of fish fed the reference diet. The wet biomass gained by fish fed diets containing 30% WCM was similar to those of fish fed the diet containing 30% ACCM and the reference diet but higher ($p=0.02$) than that of fish fed the diet containing 10% ACCM. The wet biomass gained by fish fed the reference diet was similar to those of fish fed diets containing 10 and 30% ACCM. The PER of fish fed the reference diet was similar to that of fish fed the diet containing 30% WCM but higher ($p<0.01$) than those of fish fed diets containing 10 and 30% ACCM. The PER of fish fed diets containing 30% WCM and 10% ACCM were similar but higher than that of fish fed the diet containing 30% ACCM. The PER of fish fed diets containing 10 and 30% ACCM were similar. The ADP per fish fed the diet containing 30% WCM was similar to that of fish fed the diet containing 30% ACCM but higher ($p=0.02$) than those of fish fed the reference diet and the diet containing 10% ACCM. The ADP per fish fed the reference diet was similar to those of fish fed diets containing 10 and 30% ACCM. The ANPU per fish fed the diet containing 30% WCM was similar to that of fish fed the reference diet but higher ($p=0.01$) than those of fish fed diets containing 10 and 30% ACCM. The ANPU of fish fed the reference diet was similar to those of fish fed diets containing 10 and 30% ACCM. Whole-body protein content of wet fish fed the diet containing 30% WCM was similar to those of fish fed diets containing 10 and 30% ACCM but higher ($p=0.01$) than that of fish fed the reference diet. Whole-body protein of wet fish fed the diet containing 10% ACCM was similar to that of fish fed the reference

diet. Whole-body ash ($p=0.85$) and moisture ($p=0.73$) contents of wet fish did not differ among dietary treatment.

Some organo-somatic indices and blood parameters (Table 6.4, Figures 6.1-3) varied among dietary treatments. The VSI ($p=0.50$) and SSI ($p=0.32$) were similar among dietary treatments. The HSI of fish fed the reference diet, 10% and 30% ACCM were similar but larger ($p<0.01$) than that of fish fed the diet containing 30% WCM. The VFSI did not differ ($p=0.26$) by dietary treatment. Fish fed 10% ACCM had a similar volume of packed RBCs (Hk) to fish fed the reference diet and the diet containing 30% ACCM but higher ($p=0.01$) than that of fish fed the diet containing 30% WCM. The Hk of fish fed diets containing 30% WCM and 30% ACCM were similar to that of fish fed the reference diet. The Hb content of fish fed diets containing 10 and 30% ACCM (Figure 3) were similar but higher ($p<0.01$) than those of fish fed reference diet and the diet containing 30% WCM. The Hb of fish fed the reference diet was similar to that of fish fed the diet containing 30% WCM. Mean corpuscular hemoglobin content of RBCs in blood was not altered ($p=0.22$) by dietary treatments. Lysozyme activities per mL of serum after 15 ($p=0.72$) or 30 ($p=0.99$) mins were similar among dietary treatments. There were also no differences ($p=0.49$) in ACH50 among dietary treatments.

Discussion

Weight of HSB increased with increasing carinata meal in the diets. Increased weight gain was attributed to increased consumption and relative growth rate. SGR associated with weight gain. The high consumption of the diet containing 30% WCM resulted in high intake of protein that was efficiently converted into fish protein and resulted in higher K values of fish fed 30% WCM. Although the feed conversion of HSB

fed the reference diet was similar to that of the diet containing 30% WCM, the lower feed consumption resulted in lower K values for HSB fed the reference diet. The poor feed conversion of diets containing 10 and 30% ACCM resulted in lower K values, even at higher feed consumption. Differences in growth could have also been due to differences in the lysine content of the diets. All diets were formulated to meet known minimum essential amino requirements of HSB (NRC 2011) based on analyzed amino acids of processed carinata meals, however, lysine concentrations increased with increasing carinata (1.61-2.19%) in the diets.

There was no lethal effect of processed carinata meals on HSB as shown by the high survival among dietary treatments. Cheng et al. (2010) reported mortalities of Japanese Sea Bass *Lateolabrax japonicas* fed diets containing 40 and 50% canola meals with 6.53 and 8.40 μ moles of GLS per gram of diet, respectively. Reduced survival was also reported in Rainbow Trout *Oncorhynchus mykiss* (RBT) fed diets containing $\geq 20\%$ of the diets as albumin and globulin rapeseed protein fractions (Nagel et al. 2012). That study reported GLS contents of <0.1 and 2.31 μ moles per gram of albumin and globulin protein fraction, respectively. They did not detect GLS in diets containing albumin fractions at 27.5 and 36.7%, and 0.23, 0.47 and 0.58 μ moles of GLS per gram of diet were reported in diets containing globulin fractions at 20, 30 and 40%. However, Burel et al. (2001) did not report any mortalities in RBT fed diets containing incremental amounts of rapeseed meal (RSM) up to 30% of the diet. The analyzed GLS in those diets provided up to 7.3 μ moles per gram of diet. Based on these above observations, GLS and / or their break down products in diets may not explain the observed mortalities.

Consumption would be expected to decrease with increasing carinata in diets because GLS and breakdown products (Fenwick et al. 1982; Mithen et al. 2000); and sinapine (Butler et al. 1982) impart a bitter taste. However, carinata meals used in the current study were processed to reduce concentrations of those compounds. Processing of both ACCM and WCM involved some form of meal washing with water. Glucosinolates are water-soluble and can directly leach into water (Volden et al. 2009), which enables them to be removed with the centrate during centrifugation to separate solids from the liquid. Water also facilitates the hydrolysis of GLS by myrosinase (Parkin 2008). The primary GLS in carinata is sinigrin (Matthaus and Angellini 2005), for which the breakdown product allyl isothiocyanates are volatile (Dai and Lim 2014) and may have evaporated during processing. Sinapine is also water soluble (Tan et al. 2011) and would be lost in the centrate as well. Additionally, heating and drying during meal processing and feed manufacturing increase the volatilization of isothiocyanates (Price et al. 2005) and sinapine (Zeb et al. 2006). No GLS breakdown products were detected in diets and sinapine was not analyzed in this study. Also, consumption did not decrease with inclusion of processed carinata meals in diets because the same amount of animal meals and fish oil were used in all diets and the amount of plant meals (63.6-65.6%) was fairly similar.

The FCR of HSB fed the reference diet and the diet containing 30% WCM were lower because of the combined effect of PER and ANPU. The amount of protein consumed would be expected to associate with the amount of feed consumed because the targeted dietary protein was equivalent. However, the small differences in the analyzed protein accounts for increased differences in protein consumed already established by

feed consumption. The trend in wet biomass gained by HSB associated with the trend in growth because survival was similar among diets. Differences in PER associated with differences in ANPU. Differences in APD among diets would be explained by a combination of differences in the protein consumption and ANPU. Differences in ANPU among the treatments can be explained by the essential amino acid composition of the diets. Whole body wet protein contents increased with increased carinata meal in the diets associating with the amount of protein intake.

HSB fed diets containing 30% WCM had smaller livers most likely because WCM had less available carbohydrate. Rawles and Gatlin (1998) reported increase in HSI in HSB with increasing soluble carbohydrate, which Woods et al (1995) attributed to increased fluid accumulation in the liver with increased dietary carbohydrate. Fluid accumulation is due to glycogen binding with water (Lebret et al. 1999). The two wash steps involved in processing the WCM likely reduced soluble carbohydrates more than the single wash step in the ACCM.

The Hk and Hb contents of RBCs were high in HSB fed the ACCM most likely because of its high iron content. ACCM is higher in iron (Table 3.2) than wheat gluten, whole wheat, corn (NRC 2011) and Empyreal75 (AAFCO 48.89 corn protein concentrate, Cargill Inc.) that were used in the current study at $\leq 14\%$ of the diets. The second wash step in WCM would have accounted for the difference in iron content between ACCM and WCM.

Most research on use of mustard plant meals in fish nutrition have been done with *Brassica napus* (canola/rapeseed) meals (Burel et al. 2000a, b; Webster et al. 2000; Burel et al. 2001) and, recently *Camelina sativa* (Tuziak et al. 2014; Hixson et al. 2016; Collins

et al. 2018) and carinata (Anderson et al. 2018). The only study that reports use of mustard meals in HSB was by Webster et al. (2000). They did not observe reduced growth or feed intake between HSB fed a diet containing 20% of canola meal and 27% of meat and bone meal (MBM) without FM; and HSB fed a reference diet containing 30% FM. However, the FCR (2.88) of the diet containing canola was higher than that of the reference diet (2.00). Therefore, it may be hard to draw comparisons between the current study to the Webster et al. (2000) study because the control diet (30% FM) and the diet containing canola (27% MBM) contained different animal proteins that were also slightly different in inclusion levels. Other studies on the use mustard meals in fish diets have been reported in other fish species.

Burel et al. (2000a) included 30 and 50% of dehulled RSM, processed by either pressure cooking and hexane extraction (RSM1) or double pressing and hexane extraction (RSM2), in diets of RBT and observed reduced growth at both inclusion levels due to reduced feed efficiency. However, when Burel et al. (2001) performed a similar experiment as in Burel et al. (2000a), they observed no reduction in Trout growth when rapeseed meal, processed as RSM1, was included at 30% of the diet. The FMs in the reference and test diets in both studies were similar, suggesting differences in the processing efficiencies or varieties of RSMs. Burel et al. (2000b) included RSM, processed as in Burel et al. (2000a) at 30 and 46% of RSM1 and 30% of RSM2 in Turbot *Psetta maxima* diets. Growth was reduced by RSM1 at 46% and by RSM2 at 30% inclusions in the diet, also due to reduced feed efficiency.

Hixson et al. (2016) included pressed and solvent extracted camelina meal at 7, 14 and 21% of the diets of RBT, and 8, 16 and 24% of the diets of Atlantic Salmon *Salmo*

salar. Growth was reduced by inclusion of camelina meal at 21% in RBT diets due to poor feed conversion and protein efficiency. Atlantic Salmon did not tolerate 8% of camelina meal in their diets. The FCR and PER of Atlantic Salmon did not vary with dietary treatment due to variability but the reference diet provided better performance. Tuziak et al. (2014) included camelina meal at 15 and 30% of the diets of Atlantic Cod *Gadus morhua* and observed reduced growth at 15% inclusion of camelina meal due to reduced feed intake, resulting in high feed conversions. Collins et al. (2018) processed a high oil residue camelina meal by soaking, treatment with various carbohydrases and fermentation in combination with wheat kernels using *Rhizopus oligosporus*. All processed camelina meals were included in diets of RBT at 8% of the diets. Diets had the same amount of FM but differed in the amount of blood meal and poultry by-product meal. There were no differences observed in growth after 16 weeks due to similar feed consumption, FCR and PER.

Anderson et al. (2018) replaced 28.6% of FM in a reference diet containing 35% FM and 45% total animal meals with soaked and heated (86°C for 10 mins) carinata and camelina meals (15% of the diet) in diets of RBT fingerlings. After 111 days of the study, they observed reduced growth with FM replacement with camelina meal. However, they did not observe reduced growth with FM replacement with carinata meal. There was no difference in growth between RBT fed the carinata and camelina diets. The FCRs for all diets were 0.9 and the PERs were 2.36 and 2.44 for the camelina and carinata diets, respectively.

Conclusion

Processing HE carinata seed meal reduced antinutrients in the meals, improving its nutritional value to HSB. This resulted in improved growth and feed performance. Most nutrition studies in fish have tested how much rapeseed/canola meals can replace FM at higher (>20%) inclusions of animal meals in diets, and most have resulted in reduced growth, especially with 30% inclusions of rapeseed/canola meals. However, the amount of animal meals in the current study were low (20%) but similar among all dietary treatments and the next step would be to evaluate how much FM can be replaced by processed carinata meals at similar or different animal protein ratios in diets.

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Table 6.1. Formulation (g/100g, dry basis) of diets used to test the performance of processed carinata meals in Hybrid Striped Bass^a.

Ingredients	Reference	Carinata		
		10 % ACCM ^a	30 % ACCM ^a	30% WCM ^a
Fish meal (special select) ^b	10.00	10.00	10.00	10.00
ACCM ^c	0.00	10.00	30.00	0.00
WCM ^c	0.00	0.00	0.00	30.00
Fermented soy-Gen1 Batch#3 ^c	10.00	10.00	10.00	10.00
Poultry by-product meal ^d	10.00	10.00	10.00	10.00
Empyreal 75 ^e	9.80	9.00	3.20	1.30
Wheat Midds ^f	10.00	6.96	4.63	5.18
Corn ^g	9.70	9.85	9.40	9.00
Whole cleaned wheat ^g	14.08	12.02	6.20	8.60
Wheat gluten ^h	10.00	6.30	2.00	1.50
CMC ⁱ	2.60	1.85	0.10	0.00
Vitamin premix ^f	1.50	1.50	1.50	1.50
Mineral premix ^f	1.50	1.50	1.50	1.50
Stay C ^j	0.30	0.30	0.30	0.30
Choline (60%) chloride ^k	0.30	0.30	0.30	0.30
Lysine ^l	0.20	0.20	0.20	0.10
Tryptophan ^l	0.12	0.12	0.12	0.12
Methionine ^m	0.20	0.20	0.20	0.20
Mono calcium phosphate ⁿ	1.00	1.00	1.00	1.00
Menhaden, fish oil ^b	8.00	8.00	8.00	8.00
Soybean oil ^o	0.70	0.90	1.35	1.40
Proximate composition				
Crude protein (%) ^p	43.6	44.0	44.9	45.3
Crude lipid (%) ^q	12.1	12.1	12.	12.1
Crude fiber (%) ^q	5.5	5.4	5.5	5.5
Ash (%) ^q	8.6	9.0	10.1	10.2
NFE (%) ^q	24.6	25.0	24.1	25.5
Gross energy (GE, MJ/Kg) ^q	19.89	20.07	20.21	20.05
Protein:Energy (g/MJ) ^q	21.15	21.52	21.70	21.02
Glucosinolates (μmolesg ⁻¹) ^p	0.00	0.042	0.51	0.54

^aDiets formulated to contain 44% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC; ^bOmega proteins, Houston, TX; ^cSouth Dakota State university, Brookings, SD; ^dTyson Foods Inc., Springdale, AR; ^eCargill, Blair, NE; ^fNutra Blend LLC, Neosho, MO; ^gAg First Farmers Co-op, Brookings, SD; ^hManildra Milling Corporation, Leawood, KS; ⁱAkzo Nobel Functional Chemicals BV, Amersfoort, Netherlands; ^jDSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China; ^kBiochem Corporation, New Hampton, NY; ^lAnjinomoto Animal Nutrition group, Chicago, IL; ^mAdiddeo Blue Star Company, North Point Parkway, GA; ⁿPCS sales, Northbrook, IL; ^oSouth Dakota Soybean Processors, Volga, SD; ^pAnalyzed; ^qCalculated.

Table 6.2. Analyzed amino acids composition (g/100g, dry basis) of diets used to test the performance of processed carinata meals in Hybrid Striped Bass^a.

Diet	Reference	Carinata			Requirement ^b
		10% ACCM ^a	30% ACCM ^a	30% WCM ^a	
Essential					
Arginine	1.87	1.95	2.41	2.64	1.0
Histidine	0.94	0.95	1.03	1.20	NK
Isoleucine	1.61	1.63	1.78	1.91	NK
Leucine	3.55	3.49	3.21	3.44	NK
Lysine	1.36	1.36	1.66	1.75	1.6
Methionine ^c	1.49	1.47	1.43	1.58	0.7
Phenylalanine	2.01	1.98	1.96	2.13	0.9
Threonine	1.32	1.43	1.64	1.69	0.9
Tryptophan	ND	ND	ND	ND	0.3
Valine	1.98	2.03	2.23	2.36	NK
Conditionally essential					
Cysteine ^d	1.68	1.73	1.49	2.24	0.4
Tyrosine	1.42	1.48	1.47	1.68	NK
Non essential					
Alanine	2.05	2.06	2.10	2.05	-
Aspartate	2.67	3.02	3.05	3.43	-
Glutamate	8.84	8.40	6.62	8.25	-
Glycine	1.88	1.86	2.14	2.30	-
Proline	3.08	2.81	2.68	2.70	-
Serine	1.75	1.78	1.79	1.83	-

^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC. ND = Not detected, NK = Not known.

^bNRC (2011). Cysteine calculated as a difference of the combined requirement of cysteine and methionine;

^cMethionine sulfoxide; ^dCysteic acid.

Table 6.3. Performance variables of Hybrid Striped Bass fed processed carinata meals^a.

Variable	Reference	Carinata			P-value
		10% ACCM ^a	30% ACCM ^a	30 % WCM ^a	
Initial weight/fish (g)	19.24±0.22	19.08±0.25	19.15±0.10	19.10±0.20	0.968
Final weight/fish (g)	93.3±2.2 ^{ab}	89.3±2.0 ^b	94.0±3.6 ^{ab}	103.0±2.5 ^a	0.011
Weight gain/fish (g)	74.1±2.1 ^{ab}	70.2±2.0 ^b	74.8±3.6 ^{ab}	83.9±2.4 ^a	0.010
Weight gain/day (g)	0.69±0.02 ^{ab}	0.66±0.02 ^b	0.70±0.03 ^{ab}	0.78±0.02 ^a	0.010
Relative growth (%)	385.1±11.1 ^{ab}	368.1±10.8 ^b	391.0±19.6 ^{ab}	439.2±11.4 ^a	0.015
SGR (%) ^b	1.49±0.02 ^{ab}	1.45±0.02 ^b	1.50±0.04 ^{ab}	1.59±0.02 ^a	0.0016
Fulton's K	1.22±0.01 ^b	1.24±0.01 ^b	1.25±0.02 ^b	1.35±0.01 ^a	<0.001
Survival (%)	100±0.00	99.17±0.83	99.17±0.73	100.0±0.00	0.721
Feed consumed (g)	2530.2±45.2 ^b	2651.8±70.7 ^{ab}	2832.6±73.6 ^a	2811.6±37.1 ^a	0.003
FCR ^c	1.71±0.04 ^b	1.89±0.03 ^a	1.92±0.06 ^a	1.68±0.03 ^b	0.001
Protein consumed (g)	1103.69±19.7 ^c	1166.81±31.1 ^{bc}	1282.03±33.3 ^a	1258.76±16.3 ^{ab}	<0.001
Wet biomass gained (g)	1481.0±42.2 ^{ab}	1421.0±46.6 ^b	1496.0±72.1 ^{ab}	1678.0±48.4 ^a	0.017
PER ^d	1.34±0.03 ^a	1.22±0.03 ^{bc}	1.17±0.04 ^c	1.33±0.03 ^{ab}	0.001
APD/fish (g) ^e	14.08±0.89 ^b	14.32±1.21 ^b	16.28±1.27 ^{ab}	18.73±0.80 ^a	0.019
ANPU/fish ^f	0.26±0.01 ^{ab}	0.24±0.01 ^b	0.25±0.00 ^b	0.29±0.01 ^a	0.011
Whole-body protein (% wet)	20.21±0.36 ^b	20.62±0.23 ^{ab}	21.38±0.18 ^a	21.61±0.09 ^a	0.010
Whole-body ash (% wet)	5.18±0.035	5.37±0.25	5.29±0.16	5.32±0.08	0.849
Whole-body moisture (%)	61.59±1.01	60.38±0.57	61.0±0.89	61.04±0.32	0.729

^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC. Values are means±SE; ^bSpecific growth rate; ^cFeed conversion ratio; ^dProtein efficiency ratio; ^eApparent protein deposition; ^fApparent net protein utilization.

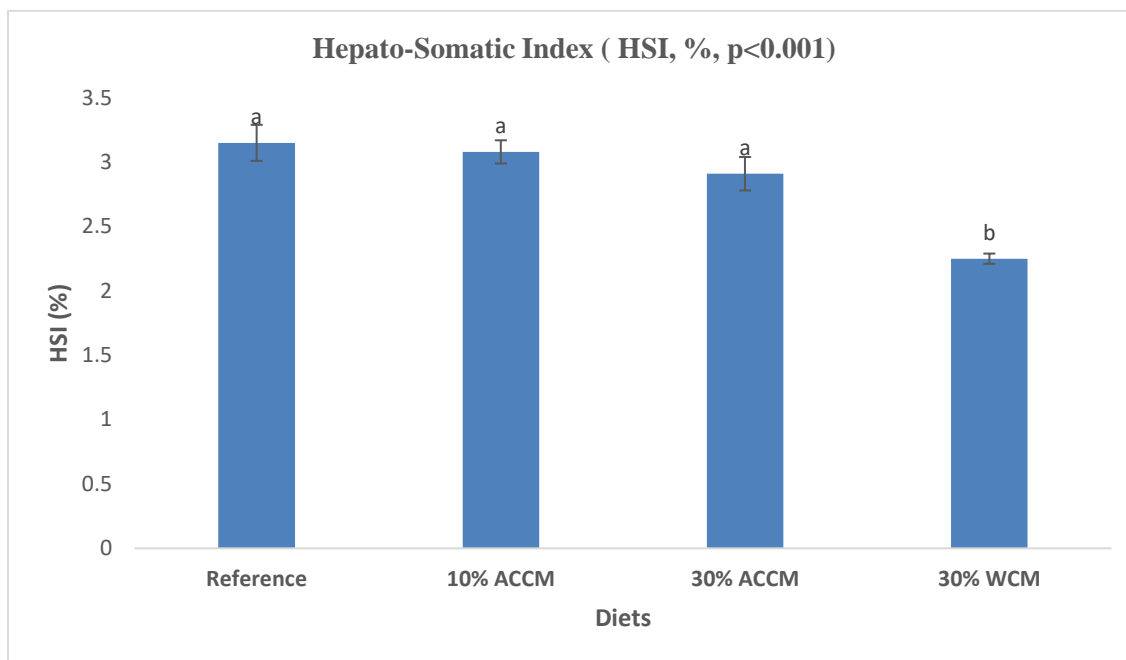
Table 6.4. Organo-somatic indices and nonspecific immune parameters of Hybrid Striped Bass fed processed carinata meals^a.

Variable	Reference	Carinata			P-value
		10% ACCM ^a	30% ACCM ^a	30 % WCM ^a	
VSI (%) ^b	9.87±0.26	9.95±0.28	10.02±0.38	9.42±0.23	0.504
SSI (%) ^c	0.0492±0.005	0.0465±0.002	0.0427±0.003	0.0389±0.005	0.322
VFSI (%) ^d	4.96±0.16	6.33±0.34	5.88±0.85	5.37±0.24	0.256
MCHC (g/dL) ^e	13.68±0.31	14.57±0.54	15.52±0.93	14.02±0.71	0.220
Lysozyme (units mL ⁻¹ min ⁻¹) After 15 mins	207.8±12.8	195.6±12.8	221.1.8±26.0	203.3±1.9	0.721
Lysozyme (units mL ⁻¹ min ⁻¹) After 30 mins	83.9±3.9	83.3±4.2	87.2±13.7	84.4±6.8	0.986
ACH ₅₀ (units mL ⁻¹) ^f	167.4±14.7	197.1±17.6	193.0±11.1	160.7±27.7	0.491

^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC. Values are means±SE; ^bViscerosomatic index; ^cSpleen somatic index; ^dVisceral fat somatic index, ^eMean corpuscular hemoglobin content;

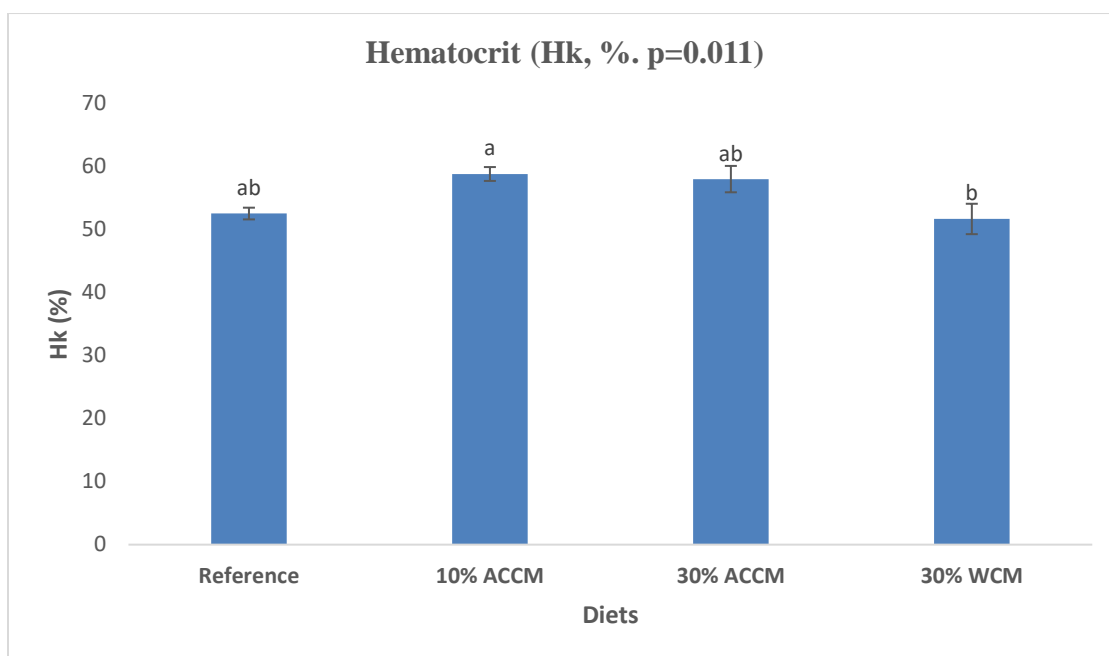
^fAlternative complement hemolysis.

Figure 6.1. Hepatosomatic index (HSI, %) of Hybrid Striped Bass fed processed carinata meals^a



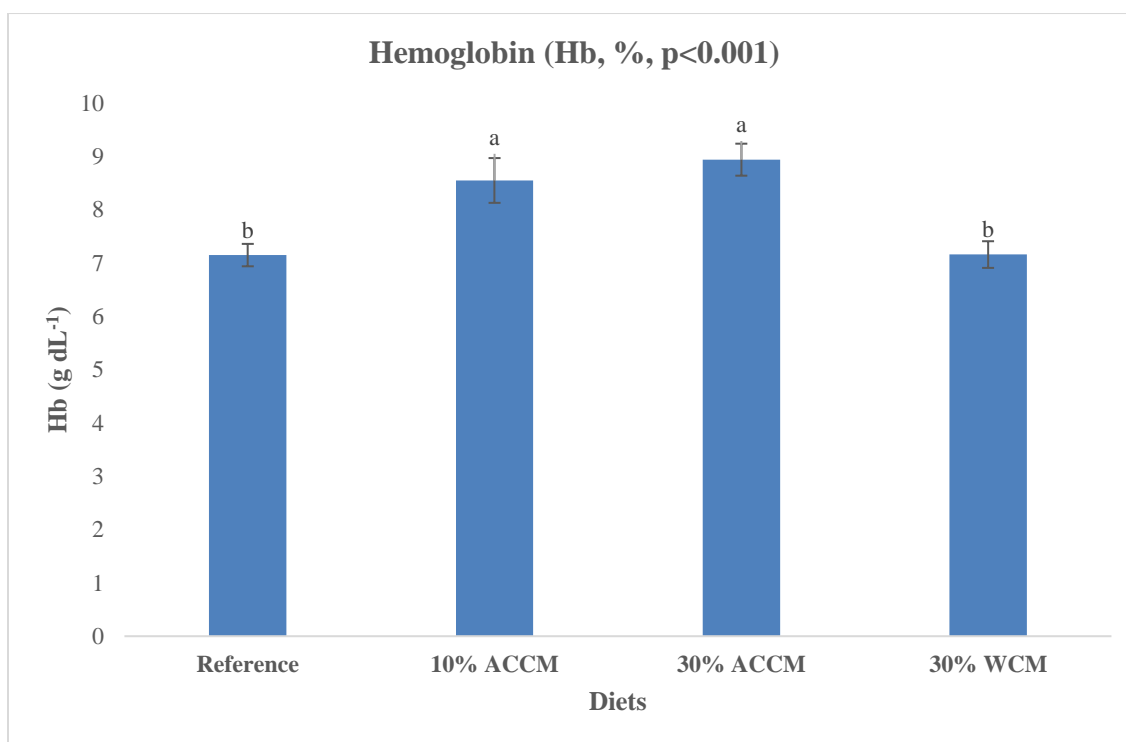
^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC. Values are means \pm SE.

Figure 6.2. Hematocrit (Hk, %) of Hybrid Striped Bass fed processed carinata meals^a



^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC. Values are means \pm SE.

Figure 6.3. Hemoglobin (Hb, g/dL) of Hybrid Striped Bass fed processed carinata meals^a



^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC. Values are means±SE.

CHAPTER 7

EFFECTS OF CARINATA *Brassica carinata* ON TRYPSIN ACTIVITY, PROTEIN AND AMINO ACID DIGESTIBILITY AND BIOAVAILABILITY IN RAINBOW TROUT *Oncorhynchus mykiss*.

Abstract

In an earlier growth trial (Chapter 5), Rainbow Trout were fed a fish meal (FM, 20%) reference diet containing 25, 50 or 75% of aerobically converted carinata meal (ACCM). Results showed that replacements of more than 25% FM reduced fish growth, partly due to reduced feed consumption. However, feed utilization was unaffected by diet. The objectives of the current study were to determine apparent digestibility coefficients (ADCs) and bioavailability of ACCM. Replacement of FM with ACCM did not alter trypsin activity or protein ADCs. Replacement of more than 25% FM reduced ADCs of arginine, histidine, isoleucine, leucine, phenylalanine, threonine, valine and tyrosine. Most amino acid ADCs for diets having more than 25% FM replaced by ACCM, were lower than protein ADCs. Lower ADCs of arginine, histidine, isoleucine, leucine, phenylalanine, threonine, valine and tyrosine in ACCM diets were not reflected in serum. Serum from RBT fed 75% ACCM had lower lysine concentrations. ACCM diets had reduced essential amino acid (EAA) peak concentrations and resulted in a slower release of EAAs in serum. Cumulative total EAAs in serum also decreased with ACCM inclusion. The pattern of total EAAs in serum for most sampling intervals associated better with that of muscle EAAs for the reference diet and diets in which 25 and 75% of FM was replaced with ACCM. Ratios of EAAs to lysine showed that tryptophan was the most limiting EAA. However, isoleucine, leucine, methionine and

phenylalanine were also inadequate for muscle synthesis for the first 9-12 hours after force-feeding. Optimal time for protein synthesis was 36 or more hours because all EAAs were adequate except for isoleucine in the muscle of RBT fed the 50% ACCM diet.

Introduction

Proteins are given first priority when formulating fish feeds not only because proteins comprise the majority of fish tissue and have roles in other metabolic activities, but because protein is the most expensive component of complete feeds (De Silva and Anderson 1995; Wilson 2002). Attempts by fish nutritionists in the last few decades to replace fish meal (FM) in fish feeds (Barrows and Sealey 2015) with various plant meals (Gatlin et al 2007) as protein sources have faced challenges; various antinutritional factors (ANFs) in plant meals reduce feed intake and nutrient utilization (Francis et al. 2001; Gatlin et al 2007; Krogdahl et al. 2010). Feed attractants may increase intake of feeds containing plant meals (Trushenski et al. 2011; Kader et al. 2012) but do not improve nutrient utilization. However, reduction of ANFs by meal processing improves both feed intake and nutrient utilization (Gatlin et al. 2007). Different plant meal processing methods have different efficiencies and therefore an understanding of fish physiological responses to processed meals is necessary for successful feed formulation when using novel ingredients.

Trypsin is considered the main enzyme that supports feed utilization and subsequent tissue growth (Torrissen and Male. 2000). Trypsin hydrolyzes peptide bonds adjacent to arginine or lysine (Ganong 2009) and is used in digestion of dietary proteins and activation of other proteolytic or non-proteolytic zymogens secreted by the pancreas such as chymotrypsinogen, proelastases, procarboxypeptidase, procolipase and

prophospholipase A (Halfon and Craik 1998). Fish may respond to FM and / or animal meal replacement by plant meals in feeds by altering trypsin production (Santiagosa et al. 2008), which will alter protein digestibility.

Protein in feedstuffs has been quantified as a measure of nitrogen since 1930s (Jones 1931; Mariotti et al. 2008). However, there are numerous nitrogen sources in a given feedstuff such as nucleic acids, phospholipids, urea, ammonia, etc., in addition to amino acids (Mariotti et al. 2008). Therefore, protein digestibility may not give an accurate measure of amino acid digestibility and bioavailability. Animals require amino acids to synthesize proteins (Wu 2009). Therefore, amino acid digestibility and bioavailability studies need to be conducted to have a thorough understanding of feeding value of a given feedstuff. The potentially lower protein and amino acid digestibility of plant meals than FM and /or animal meals (NRC 2011) is likely to reduce and / or slow amino acid digestibilities of diets containing plant meals, reducing the availability of amino acids for metabolism. Differences in available amino acids will likely lead to differences in fish growth. Therefore, amino acid digestibility and bioavailability studies may also be useful in estimating the amount of essential amino acids to be supplemented to feeds to counteract inadequacies caused FM replacement by plant meals.

Meal composition analysis, and palatability and digestibility trials (Chapter 3) were completed to evaluate processed carinata *Brassica carinata* meals (CM) as novel protein sources in Rainbow Trout *Oncorhynchus mykiss* (RBT) feeds. De-oiled (<1% oil) CM with hulls containing 48% crude protein (Table 3.1), was 92.4% digestible (Table 3.7) and had similar leucine but more arginine, histidine, methionine, threonine, tryptophan and cysteine content (Table 3.3) than solvent extracted (SE) soybean meal

with hulls (SE-SBM, NRC 2011). However, because of the negative effect of glucosinolates (GLS) and their breakdown products on long term diet palatability (chapter 4), the meal was not suitable for long term feeding trials without further processing. Subjecting de-oiled CM to aerobic conversion (AC) to reduce GLS (84%) also increased crude protein from 48 to 57% (Table 3.1) and all essential amino acids (Table 3.3) were improved except for tryptophan. However, the protein digestibility of ACCM decreased to 78% (Table 3.7) due to its high (14%) crude fiber content. The protein content of ACCM was lower than that of menhaden FM and only the contents of arginine, phenylalanine, tryptophan and valine were comparable to those of FM (NRC 2011).

Based on the above observations, a separate batch of hexane extracted CM was subjected to AC to produce (ACCM), which was used to replace FM at 0 (ref), 25 (5% ACCM), 50 (10% ACCM) and 75% (15% ACCM) in RBT diets used in the growth trial (Chapter 5). Replacement of >25% of FM by ACCM in diets of RBT containing 20% FM as the sole animal protein resulted in reduced growth, partly because of reduced feed consumption. However, feed conversion and protein efficiency ratios were unaffected by FM replacement. Therefore, follow-up studies were conducted after the growth trial with the objective of determining the effect of FM replacement by ACCM on trypsin activity and consequently protein digestibility and amino acid digestibility and bioavailability.

Materials and Methods

After conclusion of the growth trial (Chapter 5), the remaining RBT with an approximate tank average of 81-111g, were maintained in the same 32-tank recirculating aquaculture system (eight replicates/diet) and fed their respective diets without chronic

oxide (Table 7.1, whole cleaned wheat replacing chromic oxide). These fish were sacrificed to harvest their pyloric caeca for trypsin activity and to obtain blood and dorsal white muscle to quantify free amino acids. Temperature, pH and dissolved oxygen were monitored daily using a YSI Professional Plus meter (YSI, Yellow Springs, OH) and averaged (mean \pm SD) 16.2 \pm 0.5⁰ C, 8.3 \pm 0.2 and 5.9 \pm 0.9 mg/L, respectively. Unionized ammonia and nitrite were monitored weekly using a Hach DR 2000 spectrophotometer (Hach, Loveland, CO), averaging (mean \pm SD) 0.041 \pm 0.017 mg/L and 0.11 \pm 0.0 mg/L, respectively.

Harvesting pyloric caeca for trypsin activity assay. —After three weeks of feeding, fish were fasted for 48 hours before harvesting their pyloric caeca. Two randomly selected fish from each tank were euthanized and necropsied to remove viscera. The viscera were placed on an ice block to slow enzyme activity and the fat surrounding the pyloric caeca were removed before excision of the caeca. Caeca were pooled by tank, immediately frozen on dry ice, and later stored at -80⁰ C. Enzyme extracts were prepared as described by Silva et al. (2011). Frozen caeca were weighed, partially thawed on ice, and homogenized at 4mg/mL (w/v) in 0.01M Tris-HCl+ (containing 0.9% NaCl, pH 8.0) using a Polytron PT 2500 E homogenizer (Kinematica AG, Luzern, Switzerland). Homogenates were centrifuged at 10,000 x g in a chilled centrifuge and the collected supernatants were snap frozen on dry ice and later stored at -80⁰ C, pending analysis.

Blood and muscle sampling for free amino acids. —The remaining fish in the tanks were fed their respective diets for another three weeks. The fish were then hand graded, so that six fish of similar size were retained in each tank. Individual tank averages ranged from 165 to 255g. All fish were group weighed and starved for four

days. Diets were ground to particles <0.5 mm and mixed with 2X (w/v) water shortly before force-feeding. Fish were force-fed their respective diets by intubation, at a rate of 1.2% of the average fish weight in each tank and the time of force-feeding was recorded. Fish were serially sampled for blood and dorsal white muscle at 0, 3, 6, 9, 12, 24, 36 and 48 hours after force-feeding. One treatment tank was used per sampling interval and pooled samples from three fish provided replicates. All six fish in each tank were euthanized, their tails cut off and blood from three fish was pooled into one sample by combining 5-7 drops of blood from each fish into a single centrifuge tube. Centrifuge tubes containing blood were immediately put on ice and later kept in the fridge (4°C) for 12-24 hours before centrifugation. Blood was centrifuged at $1000 \times g$ in a chilled centrifuge for 10 mins to collect serum, which was frozen on dry ice and later stored at -80°C . From the same fish, white muscle tissue ($\sim 0.5\text{g}$) was excised from the left side of the dorsal fin of each fish, about 2.5 cm anterior to the fin. Muscle tissues from three fish were pooled into one sample in a centrifuge tube, immediately frozen on dry ice, and later stored at -80°C .

Serum was deproteinated as described by Yamamoto et al. (2005). Sulfosalicylic acid (10%) was added to an equal volume of serum to precipitate soluble proteins and the mixture was centrifuged at $10,000 \times g$ for 10 mins in a chilled centrifuge to collect the supernatant containing free amino acids. The supernatant was frozen at -80°C pending analysis. Muscle free-amino acids were extracted as described by Ogata and Murai (1994). About 100 mg of muscle tissue excised from each of the three muscle samples were pooled into one sample (~ 300 mg) and homogenized in 3 mL of 0.6N perchloric acid in 15 mL centrifuge tubes using pellet pestles. The homogenates were shaken for 30

mins at 200 rpm, after which they were neutralized with 0.25 mL of KOH (7.2M). The mixture was then centrifuged at 10,000 rpm in a chilled centrifuge for 10 mins and the supernatant containing free amino acids was collected and stored at -80°C , pending analysis.

Protein and amino acid digestibility. —Fifty RBT (300-400g) from a separate batch that had not been fed any experimental diet were stocked per 200-gallon tank of a 3-tank recirculating aquaculture system. Temperature, pH, dissolved oxygen, unionized ammonia and nitrite were monitored as previously described and averaged (mean \pm SD) $13.3\pm2.4^{\circ}\text{C}$, 7.7 ± 0.3 and 7.5 ± 1.1 mg/L, 0.023 ± 0.005 mg/L and 0.32 ± 0.11 mg/L, respectively. Fish were fed a reference diet without a tracer for seven days and then fed test diets (similar to growth trial diets but containing 0.75% chromic oxide, Table 7.1) for seven days, after which abdominal palpation was done to collect distal feces. Fish were combined in one tank after stripping and then randomly separated and stocked in the three tanks and fed a reference diet for another seven days before a new set of test diets were fed. Each test diet was fed to two tanks that functioned as replicates. Stripped feces were frozen and later freeze-dried using a Labconco Freezone 2.5 freeze dryer (Kansas City, MO) for 72 hours.

Freeze-dried feces and diets were finely ground in a coffee grinder and analyzed for crude protein (AOAC 2006, method 972.43) and chromic oxide using a method described by Cortes (1979). Amino acids were hydrolyzed from diets and feces using 6M HCl at 110°C for 24 hours, except glutamine and asparagine which are deamidated and tryptophan that is destroyed (Dai et al. 2014) together with cysteine and methionine (Bunka et al. 2009). Cysteine and methionine were pre-oxidized by performic acid to

cysteic acid and methionine sulfone, respectively, before acid hydrolysis (AOAC 1995, method 994.12).

Trypsin activity assay. —*N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA, Sigma Cat. No. B4875) dissolved in dimethylsulphoxide was used as a substrate and porcine pancreatic trypsin (Fisher Cat. No. AAJ6399309) used as a standard. Trypsin activity was measured as described by Silva et al. (2011) on four replicates in duplicates. Frozen enzyme extracts were thawed on ice and 140 µL of 0.1 M Tris-HCl+ were put in each well of a 96-well microplate. Thirty µL of enzyme extracts, blanks (0.1 M Tris-HCl+) or standards were then added, followed by 30 µL of BAPNA (8mM). The plates were incubated at 25⁰ C for 10 mins after which the optical density was read at 405nm using a Bio Tek Epoch plate reader (Winooski, VT). Enzyme activity was reported as µmoles of p-nitroaniline released per gram caeca.

Amino acid analysis. —Cysteic acid (Cat. No. AC226120100, Fisher Scientific, Fair Lawn, NJ) and methionine sulfone (Cat. No. AC439000050, Fisher Scientific, Fair Lawn, NJ) were added in the amino acid standards prepared from the stock solution (Cat No. WAT088122, Waters Corporation, Milford, MA). Free amino acids in samples or standards were derivatized using an AccQ-Tag derivatization kit (Cat. No. 186003836, Waters Corporation, Milford, MA), separated on an AccQ-Tag Ultra C18 1.7µm, 2.1x100 mm column (Cat. No. 186003837, Waters Corporation, Milford, MA) in an Acquity H class UPLC (Waters Corporation, Milford, MA) using reverse-phase chromatography and quantified using an Acquity UPLC PhotoDiode Array (PDA) detector (Waters Corporation, Milford, MA). Amino acid concentrations were reported as pmoles/µL for

deproteinated serum and muscle or as a percent of the hydrolyzed quantity of diets or feces.

Apparent digestibility coefficient (ADC, %) of protein and amino acids. —ADCs of protein or amino acids were calculated using the equation:

$$\text{ADC}_{\text{protein/amino acids}} = 1 - \frac{\text{Cr}_2\text{O}_3 \text{ in feed}}{\text{Cr}_2\text{O}_3 \text{ in feces}} \times \frac{\text{protein/amino acid content in feces}}{\text{protein/amino acid content in feed}}$$

Statistical analysis. —All statistical analyses were completed with version 3.4.3 of R statistical software (R Core Team 2017). Non-normal data was power transformed before statistical analysis. Trypsin activity and digestibility data were analyzed with One-Way Analysis of Variance (ANOVA) and Tukey's HSD test used to identify treatment differences. All serum and muscle free amino acid temporal data were analyzed using repeated measures ANOVA, except muscle free arginine that was analyzed using Friedman's test. Least square means were used for multiple comparisons. Treatment means were considered different at $P < 0.05$

Results

Trypsin activity, protein and amino acid digestibility. —There was an upward trend in trypsin activity with FM replacement with ACCM, but the increase was not statistically different ($p=0.32$) even with 75% FM replacement (Table 7.2). All FM replacements did not reduce ($p=0.76$) protein ADCs (Table 7.2). However, replacement of more than 25% of FM reduced arginine ($p<0.01$), histidine ($p=0.01$), isoleucine ($p<0.01$), leucine ($p<0.01$), phenylalanine ($p<0.01$), threonine ($p<0.01$), valine ($p<0.01$) and tyrosine ($p=0.01$) ADCs (Table 7.2). FM replacements did not alter lysine ($p=0.37$), methionine ($p=0.06$) and cysteine ($p=0.07$) ADCs (Table 7.2).

Serum free amino acids. —There was no effect of FM replacement with ACCM on the essential (EAA) and conditionally essential (CEAA) amino acid concentrations except for the lysine concentration (Table 7.3). Lysine concentrations decreased ($p < 0.01$) with FM replacement. All EAA and CEAA concentrations varied with time after force-feeding. Initial concentrations of arginine, leucine, methionine and phenylalanine were low and peaked at 9 hours after force-feeding. Concentrations of arginine fell to the lowest level at 24 hours but concentrations of leucine, methionine and phenylalanine were lowest at 36 hours. Concentrations of histidine were higher at 0 hours than at 3 hours. Initial concentrations of histidine and tryptophan were high and reduced to the lowest levels at 6 hours and then increased gradually, reaching the highest concentration at 48 hours. The initial concentrations of isoleucine were high and reduced to the lowest level at 6 hours before peaking at 9 hours, remaining relatively high at 24 and 36 hours and then peaking again at 48 hours. The initial concentrations of lysine and threonine were high and reduced to the lowest concentrations at 24 and 6 hours, respectively and never peaked after that. Initial valine concentrations were high and gradually reduced to lowest concentrations at 24 hours and then peaked at 48 hours. Cysteine concentrations peaked at 3 hours and remained high until 48 hours. Initial tyrosine concentrations were high and gradually reduced, reaching lowest concentrations at 36 hours and remained low at 48 hours.

Muscle Free Amino Acids. —Replacement of more than 25% FM reduced histidine concentrations (Table 7.4). Histidine concentrations also slightly peaked at 6 hours and remained relatively similar after 6 hours. Lysine concentration of RBT fed 5% ACCM was similar to that of RBT fed the reference and 10% ACCM diets but higher

than that of RBT fed 15% ACCM. Lysine concentrations of RBT fed the reference, 10% ACCM and 15% ACCM diets were similar. Initial lysine concentrations of all diets were high and remained high until 9 hours, reducing to the lowest concentrations at 24 hours and remained low after 24 hours. FM replacement increased threonine concentrations but the increase was not proportional to ACCM inclusion in the diets. However, threonine concentrations did not vary with time after force-feeding.

There was no dietary or time effect on concentrations of arginine, phenylalanine and tyrosine (Table 7.4). There was also no dietary effect on concentrations of isoleucine, leucine, methionine, or valine. However, concentrations of these amino acids varied with time after force-feeding. Initial isoleucine concentrations were high and reduced to the lowest concentration at 6 hours before peaking again at 9 hours and remained relatively high after 9 hours. Concentrations of leucine increased gradually peaking at 9 hours and then gradually reduced, reaching a low level at 36 hours. Concentrations of methionine increased gradually peaking at 9 hours and remained high after 9 hours. Initial valine concentrations were high and gradually reduced, reaching the lowest level at 6 hours and then gradually increased, peaking at 48 hours. Tryptophan and cysteine concentrations were below the detection limit (5.5 nmol/g of muscle) for most sampling times.

Discussion

Trypsin activity, protein and amino acid digestibility. —Trypsin activity did not change with FM replacement because carinata contains insignificant amounts of trypsin inhibitors (≤ 2.4 TIU/g, Bell and Rakow 1996) compared to soybean (62,081 TIU/g, Pisulewska and Pisulewska 2000; 43-84,000 TIU/g, Guillamon et al. 2008), peas (3,673 TIU/g, Pisulewska and Pisulewska 2000), and camelina (10,800-13,400 TIU/g, Almeida

et al. 2013). Therefore, no inclusion level of carinata meal in diets could contribute enough trypsin inhibitors to reach the potentially limiting concentration of 5mg of trypsin inhibitors/g of diet in RBT (Krogdahl et al. 1994). Raw wheat and corn contain 4.7 and 7.5mg of trypsin inhibitors/g of meal, respectively (Venou et al. 2003) and would have to be fed almost 100% to RBT to have some effect on trypsin activity. However, concentrations of trypsin inhibitors may increase in their glutens since glutens are processed by isolating starch from protein, which concentrates the remaining proteins (Day et al. 2006).

Trypsin inhibitors bind trypsin (Savage and Morrison 2003), resulting in alterations in trypsin activity but the effect depends on the amount of trypsin inhibitors in diets (Krogdahl et al. 1994; Olli et al. 1994). Trypsin activity may increase at relatively lower trypsin inhibitor contents of diets as a compensatory mechanism, however, at higher concentrations of trypsin inhibitors in diets, the ability to synthesize trypsin breaks down, possibly due to pancreatic exhaustion, resulting in low trypsin activity (Krogdahl et al. 1994; Olli et al. 1994).

Changes in trypsin activity in fish intestines are studied when an ingredient such as soybean meal containing trypsin inhibitors is included in fish diets. However, phytic acid was earlier demonstrated to also reduce trypsin activity *in vitro* (Singh and Krikorian 1982) and later in sows (Mroz et al. 1995). The same effects were not achieved when pure phytates were included in diets of Atlantic Salmon *Salmo salar* up to 1.0% of the diet (Sajjadi and Carter 2004). Trypsin activity in the current study did not change with increasing ACCM in diets although carinata meals contain phytates (Table 3.5).

The TIU/g of caeca observed in the current study (3.84-4.72 TIU min⁻¹ g⁻¹) are slightly higher than Heidarieth et al. (2013) reported (3.2-3.9 TIU min⁻¹ g⁻¹). The difference could be because they assayed whole intestines as opposed to the pyloric caeca assayed in the current study, yet trypsin activity is higher in the proximal intestine and pyloric caeca, which are active sites of serine proteases from the pancreas (Guillaume and Choubert 2001). In addition, differences in trypsin activity would be due to minor differences in fish size as reported in Atlantic Salmon *Salmo salar* (Torrisen et al. 1994).

Similar activity of trypsin could have contributed to similar protein ADCs even with the highest replacement of FM in diets. The potentially low protein ADC (~78%, Table 3.7) of ACCM could have been compensated for by the higher protein ADC of corn gluten, which increased with increasing ACCM in diets. The protein ADC of corn gluten (92%) is slightly higher than the protein ADC (90%) of menhaden FM (Barrows et al. 2015). Also diets with more ACCM had less whole wheat, whose protein ADC (85%) is less than menhaden FM, and corn gluten protein ADCs (Barrows et al. 2015). Another possible explanation would be the under-estimation of the protein ADC of ACCM because diets were cold extruded, yet extrusion is reported to improve protein ADCs in some plant meals (Booth et al. 2000; Cheng and Hardy 2003). The protein ADC of the cook-extruded reference diet was 85% (Barrows et al. 2015), which is 5.8% higher than what we obtained (79.2, Table 3.7). If a correction factor of 5.8% is applied to the protein ADC of ACCM, the protein ADC of ACCM would be about 84.5%.

The ADCs of arginine, histidine, leucine, phenylalanine, threonine, valine and tyrosine of corn gluten are close to those of menhaden FM but that of isoleucine is less than that of FM (Barrows et al. 2015). ADCs of arginine, histidine, isoleucine, leucine,

phenylalanine, threonine, valine and tyrosine of whole wheat are less than the ADCs of similar amino acids in FM (Barrows et al. 2015). Since whole wheat reduced with increasing ACCM in diets, the low ADCs of arginine, histidine, isoleucine, leucine, phenylalanine, threonine, valine and tyrosine with increasing ACCM in diets is most likely due to low ADCs of those amino acids in ACCM.

Reduction of ADCs of all EAAs below protein ADCs for 10 and 15% ACCM diets except for lysine and methionine (10% ACCM) could be due to differences in digestibility of other nitrogenous compounds in feed ingredients. Barrows et al. (2015) also observed differences between protein ADCs and average ADCs of amino acid irrespective of whether the ingredients were of animal or plant origin.

Serum free amino acids. —Lysine concentrations in serum decreased with increasing ACCM in diets although there were no effects of ACCM inclusion in diets on lysine ADC. This may have been caused by the low lysine content of the 15% ACCM diet (Table 5.2). However, reduction in ADCs of arginine, histidine, isoleucine, leucine, phenylalanine, threonine, valine and tyrosine of the 10 and 15% ACCM diets did not result in reduction of the concentration of these amino acids in serum.

The inability for differences in amino acid ADCs to be reflected in serum could be because blood samples were not obtained from the same fish but rather by serial sampling of different fish from the same dietary treatment. Sampling different fish may result in a variation within a treatment (Ok et al. 2001), due to individual variation among fish, which makes detecting differences among dietary treatments more difficult. However, the variation among individual fish when blood samples are obtained by serial slaughter, does not affect the pattern of amino acids over time. Ok et al. (2001) observed

a similar pattern of amino acids over time in blood samples obtained by serial sampling of blood from the caudal vasculature of different fish and blood obtained from the dorsal aorta by cannulation. However, blood samples obtained by dorsal aorta cannulation had a higher concentration of most amino acids, for which they suggested that it was probably due to the stress of repeated blood sampling of the same fish. Earlier reports showed that stress led to increased protein catabolism and thus elevated plasma free amino acids (van der Boon et al. 1991; Vijayan et al. 1991).

Amino acid ADCs may not reflect amino acid concentrations in serum post-hepatic at a given sampling time, because amino acid ADCs were obtained after absorption of amino acids from the digesta, following a gut transit time of ~8-24 hours. In preliminary studies, we fed RBT a single meal containing chromic oxide after 48 hours of starvation to determine when fecal material could reach the distal intestine. Two fish were stripped hourly starting at 6 hours until 12 hours and we observed that marked feces started appearing after 7-8 hours. Some of the remaining fish were necropsied at 24, 36 and 48 hours to determine if there was still digesta in their guts and we observed that some fish still had digesta in the distal intestine after 24 hours. However, all fish had egested the fecal material after 36 hours.

Amino acid ADCs may also not reflect concentrations of similar amino acids in serum post-liver because a significant amount of amino acids in the hepatic portal vein are removed by the liver (Karlsson et al. 2006). The percentage of amino acids removed by the liver of RBT from hepatic portal vein blood on the first pass after absorption is not well documented. However, in humans, it has been estimated that about 20-50% of amino

acids in hepatic portal vein blood are removed by the liver (Hoerr et al. 1991; Biolo et al. 1992; Hoerr et al. 1993; Matthews et al. 1993) on the first pass.

Amino acids in hepatic portal vein blood have been reported to be better indicators of dietary amino acids than amino acids in dorsal aorta blood (Karlsson et al. 2006) and most likely would have better reflected dietary amino acid ADCs observed in the current study. However, we could not obtain blood samples from the hepatic portal vein because of the small size of fish. Another missing link is that studies that have monitored amino acid changes in the hepatic portal vein and dorsal aorta blood after a meal have not linked such changes to amino acid ADCs to explain how changes in amino acid ADCs affect changes in amino acids in hepatic portal vein or dorsal aorta blood.

Blood obtained from the caudal vasculature is a combination of arterial and venous blood whose amino acid composition is intermediate between the composition of amino acids in arterial or venous blood. Therefore, dietary effects of FM replacement by ACCM on amino acid ADCs were likely not reflected in serum because the lower concentration of amino acids in venous blood diluted amino acids in arterial blood in the combined blood sample we obtained from the caudal vasculature. Concentrations of amino acids in venous blood are lower because amino acids in arterial blood are removed by extra-hepatic tissue before blood enters the veins to return to the heart. OK et al. (2001) observed that concentrations of most amino acids obtained by repeated sampling of the same fish through dorsal aorta cannulation were higher than concentrations of similar amino acids obtained from the caudal vasculature.

Despite the dilution of arterial blood by venous blood in our blood samples, amino acids in serum showed peaks of absorption, which is most likely because the

continuous absorption of amino acids over time supplied more amino acids than were drawn from blood. Most amino acids peaked at 9 hours after force-feeding except for tryptophan. Intact amino acids in dietary proteins containing 34-51% FM in the study by Karlsson et al. (2006) peaked at 6 hours after force-feeding. However, crystalline amino acids in diets in the study by Ok et al. (2001) peaked at 4 hours after force-feeding. The difference in peak times between the two studies is because of the rapid absorption of crystalline amino acids (Schuhmacher et al. 1997; Rodehutscord et al. 2000) as opposed to amino acids in intact proteins that have to be enzymatically broken down into individual amino acids, di or tri-peptides before absorption (Verri et al. 2010).

Most amino acids in the current study peaked at a later time (9 hours) than in the two above studies because most dietary amino acids were in an intact form in protein and also diets contained $\leq 20\%$ FM and thus more plant meals. However, most amino acids in serum in the current study peaked earlier than the 12 and 18 hours reported by Walton and Wilson (1986) and Schuhmacher et al. (1997), respectively. Walton and Wilson (1986) attributed the delay in amino acids peaking in plasma to a long starvation period (7 days) before force-feeding. However, Schuhmacher et al. (1997) observed a longer delay in peaking of amino acids in plasma even after a shorter starvation (2 days) period. Schuhmacher et al. (1993) compared the effect of 2 and 7-day starvation periods to determine the time at which amino acids peaked in plasma and observed that amino acids peaked at 6 hours after feeding, irrespective of the starvation periods. This suggests that diet quality impacts the time at which amino acid peak in plasma more than the fasting period.

Some EAAs and CEAAAs in the current study (Table 7.3) stayed high over an extended period after peaking before returning to baseline concentrations, which could be due to the slow release of amino acids in intact proteins (Tantikitti and March 1995). Most EAAs and CEAAAs returned to baseline concentrations between 12 and 36 hours. However, some EAAs never returned to baseline concentrations because initial concentrations (0 hours) were high possibly due to stress-related increase in protein catabolism, elevating plasma amino acids (van der Boon et al. 1991; Vijayan et al. 1991).

Larsen et al. (2012) observed broader peaks for most amino acids in plasma of RBT fed a diet in which 59% of FM was replaced by a mixture of plant proteins. We also made the same observation for serum EAAs from RBT fed the 5% ACCM. Although the peak of EAAs in serum from RBT fed 10% ACCM was not broad, the decline in EAAs was lower than that of EAA in serum from RBT fed the reference diet and the EAA concentrations changed little from 24 to 36 hours. The two peaks in EAAs in serum from RBT fed 15% ACCM were very small and the changes in EAAs were the lowest over 48 hours.

Reduction in total EAA in serum at 3 hours for RBT fed the reference, 10 and 15% ACCM diets (Figure 7.1) would be due to delayed gastric emptying caused by force feeding (Dos Santos and Jobling 1988). In addition, handling stress and anesthesia can reduce intestinal blood flow (Thorarensen et al. 1993; Eliason et al. 2007), lowering amino acid absorption. Yamada et al. (1981), Lyndon et al. (1993) and Larsen et al. (2012) also observed a reduction in amino acid concentrations in plasma at the first sampling following the 0-hour sampling. However, the reduction in concentration observed by Larsen et al. (2012) occurred in few amino acids. It is unclear why amino

acid concentrations of RBT fed 5% ACCM increased at 3 hours after force-feeding because fish handlings and anesthesia were similar across treatments.

Reduction in total EAA in serum at 6 and 9 hours after having risen at the previous sampling for RBT fed 5 and 15% ACCM, may be attributed to the anabolic drive in the liver. This could be because sampling times coincided with the highest protein synthesis in the liver triggered by arrival of amino acids especially EAA (Millward 1989) in the right proportions for protein synthesis. However, concentrations of amino acids in serum of RBT fed the reference diet and 10% ACCM only peaked at 9 hours, which may be suggestive of continuous protein synthesis in the liver as opposed to RBT fed the other diets. It should be noted that RBT fed 15% ACCM had a second peak of total EAA in serum at 12 hours. Larsen et al. (2012) also observed a slower release of amino acids in diets in which 59% of FM was replaced by a mixture of wheat, peas, field beans, sunflower and soybean. Cumulative total EAA reduced with FM replacement with ACCM (Figure 7.2), which better demonstrates effects of FM replacement with ACCM on concentrations of total EAAs in serum than temporal patterns of total EAAs in serum (Figure 7.1).

Muscle free amino acids. —The pattern of total EAAs in serum for most sampling intervals associated better with that of muscle EAAs for the reference, 5 and 15% ACCM diets (Figure 7.1) than for 10% ACCM. Decline in concentrations of EAAs at 6 hours in muscle for RBT fed the reference and 5% ACCM diets may not be fully explained by the anabolic drive in muscles because concentrations of total EAAs in serum were also low at that time. In addition, the increase in muscle EAAs at 6 hours in RBT fed the reference diet cannot be explained by concentrations of EAAs in serum because they did not

increase at that time. Lyndon et al. (1993) did not find associations between concentrations of total amino acids in cardiac plasma and muscle tissues. Differences in the pattern of amino acids between post-liver serum/plasma and muscle tissue is because free amino acids' move into muscle cells is not passive but regulated by active transmembrane transporters, which also enables maintenance of a high concentration of amino acid in muscles (Lyndon et al. 1993) as observed in the current study.

Poor association of dietary amino acids with free amino acids in muscles has also been observed in other studies (Lyndon et al. 1993; Carter et al. 1995; Yamamoto et al. 2000) because free amino acid pools in muscles are a combination of dietary amino acid supply and amino acids arising from protein catabolism in muscles (Cowey and Walton 1989). This may account for differences between dietary ADCs of lysine and threonine, and the concentrations of similar amino acids in muscles. Only muscle histidine concentrations in the current study (Table 7.4) associated with dietary histidine ADC.

We did not analyze the amino acid composition of RBT muscles in the current study because amino acid ratios to one another are not expected to change for a given species or strain of fish with diet. This could also be the reason why most fish nutrition studies do not analyze muscle amino acid compositions. However, using the amino acid composition of the fillet of RBT from the study by Turchini et al. (2018), we estimated the ratio of each EAA to lysine except for tryptophan where we used concentrations of tryptophan to lysine in the study by Nurhan (2007) to estimate the same ratio. The ratios came from different studies because Turchini et al. (2018) did not analyze tryptophan and Nurhan (2007) combined concentrations of methionine and cysteine, and phenylalanine and tyrosine. The calculated EAA to lysine ratios were used to calculate what

concentrations of EAA would be expected to be available in the muscle for protein synthesis. We then divided actual concentrations of EAA by expected concentrations at each sampling time to determine if the EAA was limiting (<1.0) or adequate (>1.0) for protein synthesis at that time (Table 7.5). Adequacy for protein synthesis in this scenario only meant adequacy for muscle synthesis and did not account for the requirements for maintenance of muscular activity.

We observed that tryptophan was the most limiting EAA for protein synthesis in the muscles (Table 7.5) because it was detected in muscles only at 0 and 36 or more hours, after feeding. This could be because any tryptophan that was transported into muscle cells may have been immediately used for protein synthesis due to the very low concentrations until 36 hours when concentrations were high enough not to limit protein synthesis. Yamamoto et al. (2000) did not detect free tryptophan in RBT muscle 12 hours after they were fed diets containing different levels of proteins, fat and amino acids. Lyndon et al. (1993) also observed that tryptophan was the most limiting amino acid for protein synthesis in Cod muscles because it was always in the lowest concentrations. Carter et al. (2000) observed very low concentrations of free phenylalanine and tryptophan compared to other EAAs in muscle when Atlantic Salmon *Salmo salar* were fed a low ration and they concluded that both amino acids were limiting to muscle synthesis. These above studies based their conclusions only on the low concentrations of free tryptophan in muscle compared to other EAAs. However, based on ratios of EAAs to lysine for each sampling time, we can also conclude that isoleucine, leucine, methionine and phenylalanine were also inadequate for muscle synthesis for the first 9-12 hours after force-feeding.

Optimal time for muscle synthesis was 36 or more hours because all EAA concentrations were adequate, with exception of muscle isoleucine in RBT fed the 50% ACCM diet. It is unclear why RBT muscle concentrated a lot of histidine than it would require for muscle synthesis based on its ratio to other EAAs but we can speculate that its transport mechanism into muscle cells may be different from other amino acids or its increased concentration may serve other purposes. Yamamoto et al. (2000) also observed an elevation in muscle free histidine concentrations at all levels of dietary protein when RBT fingerlings were fed various protein and fats levels in diets. Lyndon et al. (1993) observed high elevation in free arginine not histidine in Cod muscle. The similarity between arginine and histidine is that they both have basic side chains (Nelson et al. 2017). However, Carter et al. (2000) did not observe such elevation of a single EAA in Atlantic Salmon. Ratios of EAA to lysine did not show effects of FM replacement with ACCM on EAA concentrations in muscles. However, they showed that optimal muscle synthesis may not have coincided with the time (9 hours) at which most EAAs peaked in the muscles (Table 7.4). Cumulative total EAAs (Figure 7.3) in muscles decreased with increased ACCM which better shows dietary effects on EAAs in muscles than temporal patterns of total EAAs.

Conclusion

The studies showed some effects of FM replacement with ACCM that were not revealed by growth metrics. The association of EAA ADCs with serum EAAs could have been improved by sampling blood from the same fish by cannulation and /or the hepatic portal vein and dorsal aorta. The potentially low ADCs of some EAAs in ACCM may limit how much ACCM can replace FM and / other animal meals having more digestible

EAA. Therefore, ACCM requires further processing to improve its protein and EAA ADCs, and EAA availability in serum and muscle.

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Table 7.1. Formulation (g/100g, dry basis) of diets used for protein and amino acid digestibility in Rainbow Trout^a.

Ingredient	Reference 20% FM ^a	Carinata		
		5% ACCM ^a	10% ACCM ^a	15% ACCM ^a
Fish meal (special select) ^b	20.0000	15.0000	10.0000	5.0000
ACCM ^c	0.0000	5.0000	10.0000	15.0000
Yellow corn gluten ^d	19.5000	21.0000	22.3000	24.0000
Wheat gluten ^e	10.0000	10.0000	10.0000	10.0000
Whole Cleaned Wheat ^f	25.8975	23.5975	21.7475	19.4875
Brewers yeast ^g	0.2000	0.2000	0.2000	0.2000
CMC ^h	2.1000	1.4000	0.7000	0.0000
Vitamin premix ⁱ	1.0000	1.0000	1.0000	1.0000
Mineral premix ⁱ	0.0500	1.0000	1.0000	0.0500
Stay C ^j	0.2000	0.2000	0.2000	0.2000
Choline (60%) Chloride ^k	0.3000	0.3000	0.3000	0.3000
Arginine ^l	0.5200	0.5200	0.5200	0.5200
Lysine ^m	1.0000	1.0000	1.0000	1.0000
Methionine ⁿ	0.5000	0.5000	0.5000	0.5000
Cysteine ^l	0.1000	0.1000	0.1000	0.1000
Taurine ^o	0.5000	0.5000	0.5000	0.5000
Betaine ^l	0.5000	0.5000	0.5000	0.5000
Sodium chloride ^p	0.5000	0.5000	1.0000	1.6000
Potassium chloride ^q	0.8000	0.8000	0.8000	1.5000
Monocalcium phosphate ^r	0.5000	0.7500	1.3000	2.0000
Calcium propionate ^s	0.0025	0.0025	0.0025	0.0025
Sodium Bicarbonate ^f	0.2500	0.2500	0.2500	0.2500
Lecithin ^t	3.0000	3.0000	3.0000	3.0000
Menhaden, VA prime gold ^b	11.8300	12.1300	12.3300	12.5400
Chromic oxide ^u	0.7500	0.7500	0.7500	0.7500
Proximate composition				
Crude protein (%) ^v	44.3	44.1	43.6	44.3
Crude lipid (%) ^w	16.9	17.0	16.9	16.9
Crude fiber (%) ^w	3.2	3.2	3.2	3.2
Ash (%) ^w	9.7	9.6	9.6	9.6
NFE (%) ^w	25.9	26.1	26.7	26.0
Gross energy (GE, MJ/Kg) ^w	22.1	22.0	20.0	20.0
Protein:Energy (g/MJ) ^w	19.1	19.1	19.1	19.1

^aDiets formulated to contain 42% crude protein and 14% crude lipid. Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of fish meal (FM) in the reference diet containing 20% fish meal (FM); ^bOmega proteins, Houston, TX; ^cSouth Dakota State University, Brookings, SD; ^dKent Nutrition Group, Muscatine, Iowa; ^eManildra Milling Corporation, Leawood, KS; ^fAg First Farmers Co-op, Brookings, SD; ^gDiamond V Mills Inc., Cedar Rapids, Iowa; ^hAkzo Nobel Functional Chemicals BV, Amersfoort, Netherlands; ⁱNutra Blend LLC, Neosho, MO; ^jDSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China; ^kBiochem Corporation, New Hampton, NY; ^lPure Bulk, Rosenberg, OR; ^mAnjinomoto Animal Nutrition group, Chicago, IL; ⁿAdisseo Blue Star Company, North Point Parkway, GA; ^oJiangyin, Huachang Food Additives, Jiangyin Jiangsu, China; ^pCompass Minerals America Inc., Overland Park, KS; ^qPhibro Animal Health Corporation, Teaneck, NJ; ^rPCS Sales, Northbrook, IL; ^sAcros Organics, Morris Plains, NJ; ^tSolae LLC, St. Louis, MO; ^uFisher Scientific, Pittsburg, PA, ^vAnalyzed; ^wCalculated.

Table 7.2. Effects of replacing fish meal with processed carinata meal on trypsin activity (TA), apparent digestibility coefficients (ADCs, %) of protein, essential (EAA) without tryptophan and conditionally (CEAA) essential amino acids in Rainbow Trout^a.

Variable ^a	20% FM ^a	5% ACCM ^a	10% ACCM ^a	15% ACCM ^a	P-value
TA (U/mg of tissue) ^b	38.4±4.8	37.1±5.8	47.2±3.6	44.7±19	0.3150
EAA	ADCs (%)				
Protein	83.2±1.7	83.9±0.4	83.1±1.2	81.5±1.6	0.7580
Arginine	92.9±1.0 ^a	94.7±0.5 ^a	64.9±4.2 ^b	62.1±2.5 ^b	0.0005
Histidine	88.5±0.9 ^a	86.9±0.2 ^a	58.7±4.1 ^b	61.3±8.0 ^b	0.0092
Isoleucine	93.8±0.9 ^a	90.4±0.2 ^a	67.7±2.4 ^b	65.2±4.7 ^b	0.0013
Leucine	91.8±1.1 ^a	91.7±0.3 ^a	79.8±1.3 ^b	78.3±1.6 ^b	0.0008
Lysine	100.0±0	100.0±0	100.0±0	88.9±11.1	0.372
Methionine	91.0±1.0	90.8±2.6	88.3±0.8	75.0±7.0	0.0636
Phenylalanine	90.3±0.9 ^a	89.2±0.3 ^a	69.9±2.6 ^b	69.3±3.9 ^b	0.0019
Threonine	89.0±0.9 ^a	85.9±0.1 ^a	60.6±3.7 ^b	55.3±3.5 ^b	0.0006
Valine	92.3±0.9 ^a	89.1±0.5 ^a	67.0±2.8 ^b	64.6±3.7 ^b	0.0008
CEAA					
Cysteine	65.7±5.5	75.1±2.6	51.7±1.5	36.3±11.9	0.0712
Tyrosine	90.2±1.0 ^a	90.9±0.0 ^a	69.3±2.8 ^b	68.7±6.6 ^b	0.0095

^aDiets formulated to contain 42% crude protein and 14% crude lipid. The reference diet contained 20% fish meal (FM), Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of FM in the reference diet.

^bμmoles of p-nitroaniline released from *N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride per mg of pyloric caeca.

Table 7.3. Effects of replacing fish meal with processed carinata meal on serum free essential (EAA) and conditionally essential (CEAA) amino acids (nmoles/mL) in Rainbow Trout^a.

Diet ^a	EAA	Sampling times (hours)								p-values		
		0 ^{bc}	3 ^{bc}	6 ^{bc}	9 ^a	12 ^{ab}	24 ^c	36 ^c	48 ^{bc}	Diet	Time	Interaction
20% FM	Arg	450±55	417±34	408±46	640	509±12	349±0	308±23	407±1	0.4118	<0.0001	0.7695
5% ACCM		342±50	468±134	309±23	517±89	499±45	409±133	336±7	455±3			
10% ACCM		388±30	372±4	399±59	556±73	413±12	285±13	332±5	408±1			
15% ACCM		379±36	323±9	397±56	419±59	449±30	324±53	333±15	392±50			
		0 ^a	3 ^{bc}	6 ^c	9 ^{abc}	12 ^{bc}	24 ^{bc}	36 ^{ab}	48 ^a			
20% FM	His	136±20	92±12	94±13	116	103±1	107±2	97±6	122±5	0.4588	<0.0001	0.3684
5% ACCM		120±11	117±40	74±9	98±3	112±2	106±21	130±7	139±1			
10% ACCM		121±8	86±5	94±16	120±6	83±2	95±10	121±6	129±7			
15% ACCM		131±7	84±1	92±8	66±12	94±7	84±15	132±2	122±21			
		0 ^{ab}	3 ^{abc}	6 ^c	9 ^a	12 ^{abc}	24 ^{bc}	36 ^{bc}	48 ^a			
20% FM	Ile	149±21	106±4	83±16	171	101±14	93±7	90±2	141±1	0.1967	0.0010	0.8427
5% ACCM		115±22	148±45	75±9	113±27	98±8	89±28	88±11	141±5			
10% ACCM		121±12	114±9	92±2	130±32	95±6	90±6	97±0	141±16			
15% ACCM		114±10	92±10	92±16	81±18	82±10	91±22	91±3	104±23			
		0 ^c	3 ^c	6 ^{bc}	9 ^a	12 ^a	24 ^{ab}	36 ^c	48 ^c			
20% FM	Leu	274±31	267±14	295±50	557	455±13	380±30	244±3	297±3	0.4119	<0.0001	0.8680
5% ACCM		211±43	327±80	297±28	464±92	503±31	442±127	233±26	317±10			
10% ACCM		236±19	244±17	338±9	555±89	389±59	380±15	268±4	287±34			
15% ACCM		211±14	213	315±64	351±68	399±21	394±55	261±18	267±43			
		0 ^a	3 ^{ab}	6 ^{bc}	9 ^{abc}	12 ^{bc}	24 ^c	36 ^{bc}	48 ^{abc}			
20% FM ^a	Lys	288±64 ^a	229±32 ^{ab}	174±30 ^{ab}	218 ^{ab}	164±35 ^{ab}	130±13 ^{ab}	154±18 ^{ab}	283±24 ^a	0.0007	0.0005	0.6851
5% ACCM ^a		187±45 ^{ab}	294±107 ^a	115±9 ^{ab}	193±5 ^{ab}	131±17 ^{ab}	201±58 ^{ab}	151±22 ^{ab}	234±4 ^{ab}			
10% ACCM ^{ab}		248±39 ^{ab}	192±45 ^{ab}	154±38 ^{ab}	142±54 ^{ab}	122±28 ^{ab}	132±9 ^{ab}	149±1 ^{ab}	188±2 ^{ab}			
15% ACCM ^b		214±44 ^{ab}	153±0 ^{ab}	117±20 ^{ab}	106±8 ^{ab}	117±33 ^{ab}	72±18 ^b	108±0 ^{ab}	123±58 ^{ab}			
		0 ^d	3 ^c	6 ^{bc}	9 ^a	12 ^{ab}	24 ^{bc}	36 ^{cd}	48 ^c			
20% FM	Met	82±33	109±7	124±17	189 (NR)	160±26	112±6	83±4	91±10	0.4880	<0.0001	0.7272
5% ACCM		58±8	112±22	105±6	160±20	162±13	128±35	105±15	104±5			
10% ACCM		61±5	109±4	121±5	171±20	117±11	118±5	91±9	107±2			
15% ACCM		57±1	91±0	113±19	120±14	135±8	125±4	95±6	99±19			
		0 ^c	3 ^{bc}	6 ^{bc}	9 ^a	12 ^{ab}	24 ^{ab}	36 ^c	48 ^c			
20% FM	Phe	70±17	91±3	96±6	131	110±2	94±9	68±1	80±3	0.1967	<0.0001	0.5013
5% ACCM		74±6	92±29	78±4	114±3	128±4	102±20	77±11	75±5			
10% ACCM		79±7	94±2	95±12	134±0	97±0	115±12	93±5	87±12			
15% ACCM		75±3	91±2	93±12	89±11	103±3	116±8	74±7	73±13			

Table 7.3. Continued

Diet ^a	EAA	Sampling times (hours)								p-values		
		0 ^a	3 ^{abc}	6 ^c	9 ^{ab}	12 ^{abc}	24 ^{bc}	36 ^{abc}	48 ^{abc}	Diet	Time	Interaction
20% FM	Thr	138±11	99±16	84±14	193	96±4	79±6	93±16	133±3	0.1063	0.0057	0.6496
5% ACCM		153±71	120±36	58±6	110±21	107±16	105±40	96±2	148±6			
10% ACCM		117±8	111±24	76±2	120±11	79±14	65±6	120±5	113±1			
15% ACCM		109±39	64±0	85±6	76±13	80±15	64±18	112±7	96±29			
		0 ^a	3 ^{ab}	6 ^d	9 ^{cd}	12 ^{cd}	24 ^{bc}	36 ^{ab}	48 ^a			
20% FM	Trp	31±1	24±2	17±1	21	17±0	22±0	22±0	28±1	0.3962	<0.0001	0.8385
5% ACCM		26±1	25±4	13±0	17±0	19±1	19±1	26±3	28±1			
10% ACCM		27±1	25±1	18±3	20±3	17±0	24±1	26±4	29±3			
15% ACCM		25±0	24±1	19±1	14±1	16±	23±4	25±3	26±5			
		0 ^{ab}	3 ^{abc}	6 ^{bc}	9 ^{abc}	12 ^{bc}	24 ^c	36 ^c	48 ^a			
20% FM	Val	434±49	338±14	286±46	439	316±34	281±11	279±18	425±23	0.1692	0.0005	0.9494
5% ACCM		370±68	413±69	271±32	326±36	306±32	289±73	279±30	426±10			
10% ACCM		377±26	339±24	293±29	393±95	284±31	283±5	289±1	419±52			
15% ACCM		353±19	320±26	303±39	231±48	266±29	264±53	271±9	330±85			
	CEAA	0 ^b	3 ^a	6 ^a	9 ^{ab}	12 ^{ab}	24 ^{ab}	36 ^{ab}	48 ^a			
20% FM	Cys	11±2	15±2	12±0	10	12±2	14±1	12±1	15±0	0.8742	0.0111	0.6337
5% ACCM		10±2	14±1	12±2	13±1	12±2	14±3	13±1	16±1			
10% ACCM		9±0	14±1	15±0	15±0	9±0	13±2	11±1	14±2			
15% ACCM		8±0	12±1	16±1	10±2	13±1	13±3	11±0	12±5			
		0 ^{abc}	3 ^{ab}	6 ^{ab}	9 ^a	12 ^{abc}	24 ^{bc}	36 ^c	48 ^{bc}			
20% FM	Tyr	67±7	64±0	71±1	78	51±6	44±5	35±0	52±1	0.5394	0.0002	0.3033
5% ACCM		55±7	63±9	50±3	72±16	64±6	49±3	42±9	45±3			
10% ACCM		46±3	65±0	72±5	71±18	47±0	51±7	50±2	43±3			
15% ACCM		58±2	61±5	54±6	39±11	52±1	51±2	37±3	50±17			

^aDiets formulated to contain 42% crude protein and 14% crude lipid. The reference diet contained 20% fish meal (FM). Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. ACCM replaced 25, 50 and 75% of FM in the reference diet.

Table 7.4. Effects of replacing fish meal with processed carinata meal on muscle free essential amino acids (EAA, nmoles/g) in Rainbow Trout^a.

Diet ^a	EAA	Sampling times (hours)								p-values		
		0	3	6	9	12	24	36	48	Diet	Time	Interaction
1	Arg	1019±85	1163±3	793±498	358±201	140±6	283±62	217±8	196±14	0.3691	-	-
2		884±38	800±338	1036±75	163±12	223±46	122±5	194±105	163±97			
3		977±0	471±81	910±569	202±81	204±27	167±22	265±104	341±61			
4		955±103	672±322	1122±40	262±24	143±29	298±39	441±77	304±31			
		0^{ab}	3^{ab}	6^a	9^{ab}	12^{ab}	24^b	36^{ab}	48^{ab}			
1 ^a	His	4502±696 ^{ab}	5341±103 ^a	3441±389 ^{abc}	4595±1425 ^{ab}	3289±597 ^{abc}	3078±269 ^{abc}	3072±15 ^{abc}	3995±291 ^{abc}	0.0002	0.0331	0.02265
2 ^a		3115±212 ^{abc}	3665±365 ^{abc}	3829±717 ^{abc}	3999±369 ^{abc}	3170±38 ^{abc}	3770±199 ^{abc}	3839±443 ^{abc}	4126±679 ^{abc}			
3 ^b		2246±10 ^{abc}	2055±882 ^{bc}	4208±103 ^{abc}	4085±748 ^{abc}	1657±397 ^{bc}	2501±496 ^{abc}	3374±1585 ^{abc}	3127±664 ^{abc}			
4 ^b		3078±44 ^{abc}	3052±2 ^{abc}	4444±169 ^{abc}	2473±162 ^{abc}	3308±5 ^{abc}	1280±307 ^c	2833±47 ^{abc}	1662±42 ^{bc}			
		0^{abc}	3^{abc}	6^c	9^a	12^{bc}	24^{abc}	36^{bcd}	48^{ab}			
1	Ile	142±26	90±1	53±13	140±38	90±4	81±0	81±1	138±6	0.6521	0.0013	0.3834
2		117±9	125±34	76±18	143±17	93±8	82±14	86±11	111±18			
3		72±18	105±17	89±8	146±65	80±17	78±10	89±7	144±20			
4		123±13	61±10	69±3	79±14	60±11	106±19	114±9	110±34			
		0^d	3^d	6^{bcd}	9^a	12^{ab}	24^{abc}	36^{cd}	48^{bcd}			
1	Leu	237±39	186±3	203±4	384±65	293±23	294±33	200±9	277±10	0.6834	<0.0001	0.5170
2		209±16	244±59	233±19	392±42	384±43	307±50	203±20	250±19			
3		141±14	196±23	261±1	446±133	348±97	293±12	205±9	263±42			
4		207±13	137±21	226±7	269±28	369±18	372±37	275±21	242±88			
		0^a	3^a	6^{ab}	9^a	12^{bc}	24^c	36^c	48^{bc}			
1 ^{ab}	Lys	494±171 ^{abc}	563±19 ^a	325±54 ^{abc}	642±355 ^a	156±29 ^{abc}	26±21 ^{bc}	51±12 ^{abc}	190±19 ^{abc}	0.0292	<0.0001	0.0214
2 ^a		281±80 ^{abc}	512±11 ^{ab}	314±13 ^{abc}	572±41 ^a	208±107 ^{abc}	552±357 ^{ab}	48±48 ^{bc}	120±56 ^{abc}			
3 ^{ab}		283±35 ^{abc}	513±104 ^{ab}	344±69 ^{abc}	468±353 ^{abc}	20±3 ^{bc}	22±22 ^c	203±46 ^{abc}	172±30 ^{abc}			
4 ^b		461±43 ^{abc}	158±29 ^{abc}	253±8 ^{abc}	110±50 ^{abc}	173±86 ^{abc}	36±9 ^{abc}	59±19 ^{abc}	38±21 ^{abc}			
		0^c	3^{bc}	6^{abc}	9^a	12^{ab}	24^{ab}	36^{bc}	48^{abc}			
1	Met	124±21	126±10	150±7	190±30	126±2	123±2	118±2	142±0	0.8571	0.0003	0.0938
2		99±3	157±27	121±6	188±10	176±24	143±19	133±8	127±7			
3		77±19	146±3	161±3	203±60	144±17	139±25	127±17	162±18			
4		121±7	103±1	124±5	131±12	130±10	180±1	158±19	138±37			
		0	3	6	9	12	24	36	48			
1	Phe	132±8	116±17	134±2	158±14	133±11	126±8	119±14	166±13	0.9005	0.1450	0.3112
2		122±6	159±25	110±5	161±13	176±18	135±12	117±17	123±22			
3		92±22	133±2	136±12	179±54	155±22	145±43	129±9	154±26			
4		146±8	117±6	117±4	118±14	128±15	161±12	142±2	116±31			

Table 7.4. Continued

Diet ^a	EAA	Sampling times (hours)								p-values		
		0	3	6	9	12	24	36	48	Diet	Time	Interaction
1 ^b	Thr	483±70	547±23	523±120	517±73	394±81	480±11	511±127	608±139	0.0071	0.6597	0.4385
2 ^{ab}		547±14	558±67	349±70	631±24	724±96	536±133	574±72	587±33			
3 ^a		774±187	799±234	540±35	792±44	804±138	604±21	610±47	770±108			
4 ^{ab}		496±25	488±105	734±27	598±144	433±42	651±43	898±426	632±75			
		0	3	6	9	12	24	36	48			
1	Trp	93±4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	87±3			
2		45±45	43±43	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	59±17	63±24			
3		0±0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	45±45	50±50			
4		92±4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	81±7	0±0			
		0^a	3^{ab}	6^b	9^{ab}	12^b	24^b	36^{ab}	48^a			
1	Val	366±59	263±3	175±32	259±68	177±0	208±11	225±4	361±40	0.4946	<0.0001	0.4807
2		327±26	301±32	202±19	269±30	232±37	196±28	241±12	327±3			
3		233±29	280±34	212±12	303±118	220±35	214±26	207±11	354±60			
4		324±8	209±27	199±7	172±20	169±17	248±36	270±26	266±76			
	CEAA	0	3	6	9	12	24	36	48			
1	Cys	37±4	0±0	0±00	0±0	0±0	11±11	0±0	46±1			
2		40±5	0±0	13±12	0±0	18±18	0±0	0±0	27±12			
3		14±14	0±0	0±0	0±0	0±0	0±0	0±0	17±17			
4		0±0	0±0	0±0	0±0	0±0	0±0	15±15	0±0			
		0	3	6	9	12	24	36	48			
1	Tyr	203±51	189±71	181±25	206±14	177±12	188±7	215±0	274±35	0.6533	0.6263	0.5841
2		154±10	226±11	184±9	196±14	239±41	170±10	225±30	221±26			
3		161±29	181±7	205±27	251±63	202±32	196±54	171±16	234±50			
4		206±2	176±15	164±6	172±12	183±13	194±12	199±5	162±33			

Diets formulated to contain 42% crude protein and 14% crude lipid. Aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp. The reference diet contained 20% fish meal (FM, diet 1). ACCM replaced 25 (diet2), 50(diet 3) and 75% (diet 4) of FM in the diet. Each diet consisted of 2 replicates analyzed in duplicate.

Tryptophan and cysteine were not statistically analyzed because of 0 concentrations for most sampling times.

Table 7.5. Effects of replacing fish meal in Rainbow Trout diets with aerobically converted carinata meal at 0 (ref), 25 (5% ACCM), 50 (10% ACCM) and 75% (15% ACCM) on ratios of free essential amino acids (EAA) to lysine in the muscle at various sampling times (hours)^a

Diets ^a	EAA	Muscle ratios ^b	Sampling times (hours)							
			0	3	6	9	12	24	36	48
Reference	Arg	0.66 ^c	3.13	3.13	3.69	0.85	1.37	16.55	6.41	1.57
5% ACCM			4.76	2.37	4.99	0.43	1.62	0.35	6.12	2.05
10% ACCM			5.23	1.39	4.01	0.65	15.83	11.59	1.97	2.05
15% ACCM			3.14	6.43	6.72	3.63	1.25	12.42	11.28	12.09
			0	3	6	9	12	24	36	48
Reference	His	0.36 ^c	25.41	26.45	29.48	19.96	58.67	330.93	166.57	58.66
5% ACCM			30.84	19.96	33.94	19.50	42.42	19.04	222.41	95.53
10% ACCM			22.11	11.16	34.14	24.35	236.49	319.98	46.24	95.53
15% ACCM			18.61	53.70	48.94	62.85	53.27	98.02	133.04	118.53
			0	3	6	9	12	24	36	48
Reference	Ile	0.49 ^c	0.58	0.32	0.33	0.44	1.16	6.36	3.20	1.47
5% ACCM			0.84	0.49	0.49	0.51	0.91	0.30	3.61	1.87
10% ACCM			0.52	0.41	0.52	0.63	8.36	7.22	0.88	1.87
15% ACCM			0.54	0.78	0.56	1.47	0.71	5.89	3.90	5.83
			0	3	6	9	12	24	36	48
Reference	Leu	0.85 ^c	0.57	0.39	0.74	0.71	2.22	13.41	4.58	1.72
5% ACCM			0.88	0.56	0.88	0.81	2.17	0.66	4.99	2.45
10% ACCM			0.59	0.45	0.90	1.12	21.05	15.90	1.19	2.45
15% ACCM			0.53	1.02	1.06	2.90	1.83	12.07	5.47	7.51
			0	3	6	9	12	24	36	48
Reference	Met	0.34 ^c	0.74	0.66	1.37	0.87	2.39	14.02	6.76	2.21
5% ACCM			1.04	0.91	1.13	0.97	2.49	0.77	8.16	3.11
10% ACCM			0.80	0.84	1.38	1.29	21.85	18.85	1.84	3.11
15% ACCM			0.77	1.93	1.44	3.54	2.22	14.64	7.88	10.68
			0	3	6	9	12	24	36	48
Reference	Phe	0.47 ^c	0.57	0.44	0.88	0.53	1.82	10.36	4.96	1.87
5% ACCM			0.93	0.66	0.75	0.60	1.80	0.52	5.19	2.19
10% ACCM			0.70	0.55	0.84	0.82	16.94	14.17	1.35	2.19
15% ACCM			0.68	1.58	0.99	2.30	1.58	9.43	5.10	6.47

Table 7.5. Continued

Diets ^a	EAA	Muscle ratios ^b	Sampling times (hours)							
			0	3	6	9	12	24	36	48
Reference	Thr	0.50 ^c	1.97	1.96	3.25	1.63	5.09	37.33	20.07	6.46
5% ACCM			3.92	2.20	2.24	2.23	7.01	1.96	24.10	9.84
10% ACCM			5.51	3.14	3.17	3.42	83.06	55.97	6.05	9.84
15% ACCM			2.17	6.22	5.85	10.99	5.05	36.12	30.54	33.43
			0	3	6	9	12	24	36	48
Reference	Try	0.14 ^d	0.69	0.00	0.00	0.00	0.00	0.00	8.75	3.28
5% ACCM			1.14	0.60	0.00	0.00	0.00	0.00	8.82	3.72
10% ACCM			0	0.00	0.00	0.00	0.00	0.00	1.58	3.72
15% ACCM			1.43	0.00	0.00	0.00	0.00	0.00	9.74	20.95
			0	3	6	9	12	24	36	48
Reference	Val	0.56 ^b	1.32	0.84	0.96	0.72	2.02	14.34	7.82	3.41
5% ACCM			2.08	1.05	1.15	0.84	1.99	0.64	8.97	4.86
10% ACCM			1.47	0.98	1.10	1.16	20.16	17.57	1.82	4.86
15% ACCM			1.26	2.36	1.40	2.80	1.74	12.20	8.16	12.48

^aDiets formulated to contain 42% crude protein and 14% crude lipid. The reference diet contained 20% fish meal (FM), aerobically converted carinata meal (ACCM) was hexane extracted (HE) carinata meal that was aerobically converted using fungi spp; ^bRatios of EAA to lysine in fish muscle were used to calculate the optimal concentrations of EAAs for muscle synthesis relative to lysine at each sampling time. Actual concentrations of EAAs at each sampling time were then divided by the calculated optimal concentrations for muscle synthesis to obtain a ratio of each EAA relative to requirement for each sampling time; ^cTurchini et al. (in press); ^dNurhan et al. (2007).

Figure 7.1. Temporal patterns of total essential amino acids in serum and dorsal white muscle in Rainbow Trout fed diets in which processed carinata meals replaced fish meal at 0 (ref), 25 (5% ACCM), 50 (10% ACCM) and 75% (15% ACCM).

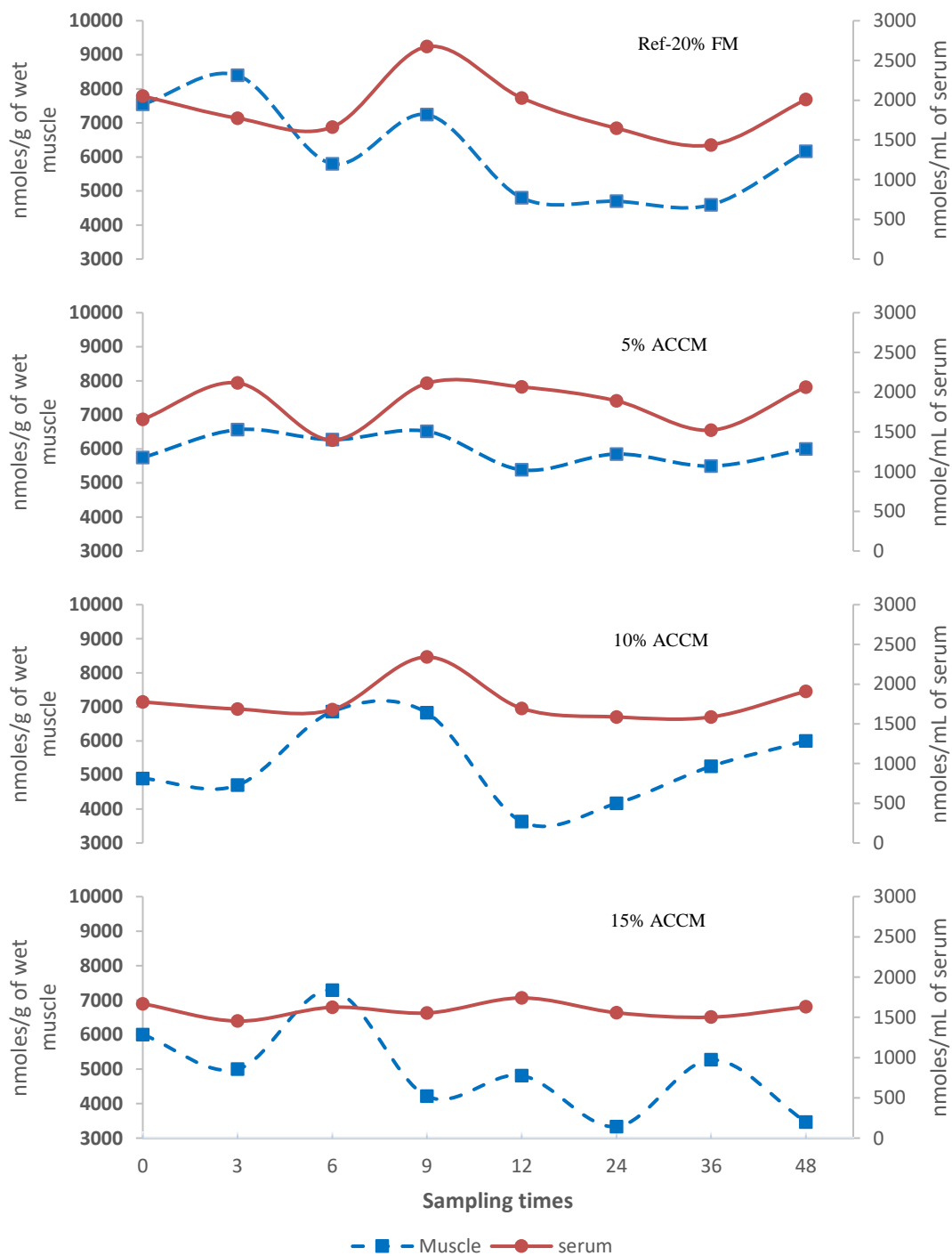


Figure 7.2. Effects of replacing fish meal with processed carinata meal at 0 (ref), 25 (5% ACCM), 50 (10% ACCM) and 75% (15% ACCM) on cumulative serum free total essential amino acids in Rainbow Trout (FEAA, nmoles mL⁻¹).

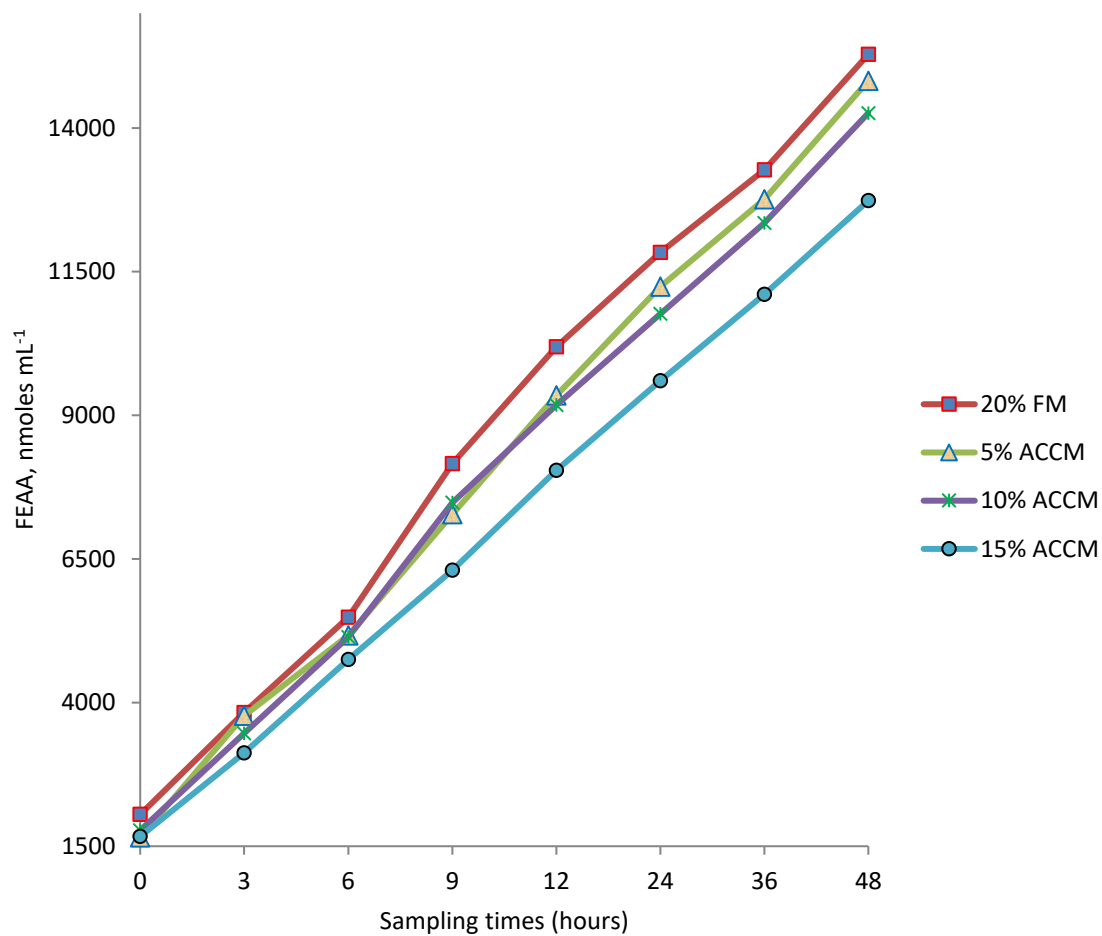
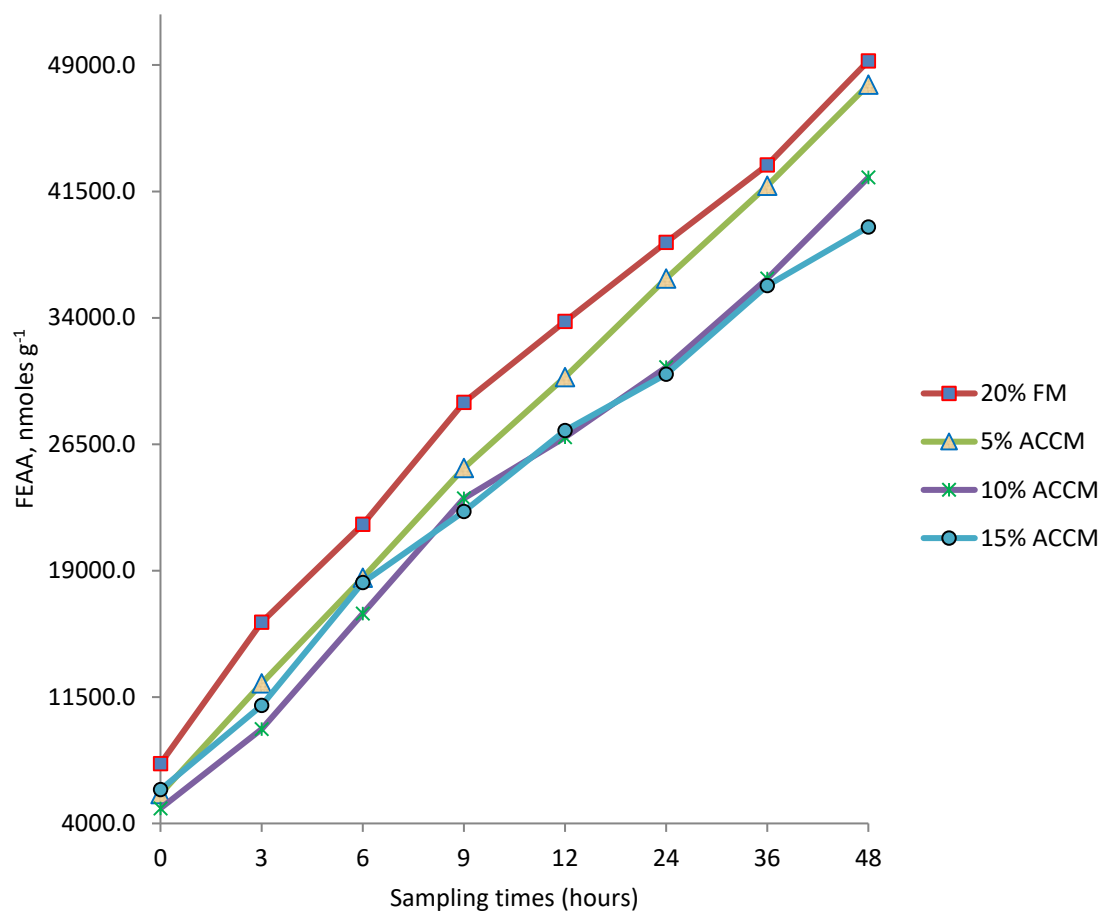


Figure 7.3. Effects of replacing fish meal with processed carinata meal at 0 (ref), 25 (5% ACCM), 50 (10% ACCM) and 75% (15% ACCM) on cumulative muscle free total essential amino acids in Rainbow Trout (FEAA, nmoles g⁻¹).



CHAPTER 8

EFFECTS OF CARINATA *Brassica carinata* ON TRYPSIN ACTIVITY, PROTEIN DIGESTIBILITY, AND AMINO ACID DIGESTIBILITY AND BIOAVAILABILITY IN HYBRID STRIPED BASS *Morone chrysops* ♀ X *M. saxatilis* ♂.

Abstract

In an earlier Hybrid Striped Bass (HSB) growth trial (Chapter 6), inclusion of up to 30% of aerobically converted (AC) carinata meal (CM) followed by a single wash (ACCM) in diets containing similar but low animal meals, did not affect fish performance primarily due to increased feed intake. However, inclusion of 30% of double washed CM (WCM) improved feed utilization in addition to fish performance. In the current study, we determined the apparent digestibility coefficients (ADCs) and availability of ACCM and WCM in HSB. Inclusion of up to 30% processed CM did not alter trypsin activity or protein ADCs. However, 30% ACCM reduced isoleucine, leucine, phenylalanine, threonine and valine ADCs. Highest ACCM (30%) reduced serum arginine and leucine. All inclusions of ACCM or WCM increased serum methionine. High inclusions of ACCM or WCM (30%) increased serum tryptophan and valine. The reference and 30% WCM diets resulted in the highest total essential amino acids (EAAs) in serum but release of total EAAs in serum of HSB fed 30% WCM was elevated continuously over a longer period. High inclusions of ACCM or WCM (30%) increased muscle histidine but resulted in lower leucine and phenylalanine. Only 30% WCM increased muscle lysine and valine. However, all inclusion levels of ACCM or WCM increased muscle methionine. High inclusions of ACCM or WCM (30%) in diets resulted in more available

total EAAs over a longer period. Muscle EAA to lysine ratios showed that only histidine concentrations were adequate for muscle synthesis over the 36-hour period.

Introduction

The percentage increase in food fish demand (3.2%) doubled the increase in human population growth (1.6%) from 1961-2013 due to an increase in per capita fish consumption from 9.9 kgs in 1960s to 19.7 kgs in 2013. With relatively constant fish production from capture fisheries from 1985 to 2014, commercial aquaculture has rapidly grown, increasing the contribution of aquaculture to total fish production from 9.3% in 1985 to 44.1% in 2014 to satisfy the increased food fish demand (FAO 2016). Increased aquaculture production has been likely supported by a corresponding increase and improvement in fish feed manufacturing and production systems. However, the Food and Agriculture Organization (FAO) of United Nations does not collect data on quantities of fish feeds manufactured by member countries (Tacon 1997). The available global fish feed production data collected by independent groups such as Alltech (Nicholasville, KY) from feed mills, omits quantities of feed made on-farm and used by small-scale farmers, especially in developing countries. This is likely why low quantities (37.3 MMT) of global fish feed production were reported for 2017 (Alltech 2018). This would be realistic only if the quantity of fed fish produced in 2017 reduced from 51.1MMT reported in 2014 (FAO 2016).

Traditionally, fish meal (FM) is the main protein source in fish feeds, especially for carnivorous fish species (Tacon et al. 2011). However, FM prices have increased in the recent two decades due to demand for fish feed which has increased with increasing aquaculture production. Pricing and fluctuating availability has forced fish nutritionists to

replace most FM in fish feeds with cheaper plant meals such as oilseed meals or other animal meals (Tacon et al. 2006; Gatlin et al. 2007; Tacon and Metian 2008) except for critical stages of fish production such as the hatchery (FAO 2016). The demand for oilseeds has increased their global production in the last two decades (Hardy 2010) and there is potential for increased production of other oilseeds such as carinata *Brassica carinata* that is undergoing genetic modification to improve its oil composition for jet-fuel production (Taylor et al. 2010). Oil extraction from carinata seeds will provide a meal that can be used in animal feeds.

We conducted traditional compositional analyses, diet palatability and digestibility (Chapter 3), in addition to a growth trial (Chapter 6) to evaluate the nutritional value of carinata meal (CM) to Hybrid Striped Bass *Morone chrysops* x *M. saxatilis* (HSB). De-oiled (<1% oil) CM with seed coats contained 48% crude protein (Table 3.1), which was 98.9% digestible (Table 3.8). It also contained similar leucine but more arginine, histidine, methionine, threonine, tryptophan and cysteine content (Table 3.3) than solvent extracted (SE) soybean meal with hulls (SE-SBM, NRC 2011). However, a previous study (Chapter 4) showed that glucosinolates (GLS) and their breakdown products in cold pressed CM, negatively affect long-term diet palatability (Chapter 4), making it unsuitable for long-term feeding trials without further processing. Therefore, we subjected CM to aerobic conversion (AC) followed by a single wash (ACCM). The GLS content was reduced by 84%, crude protein increased from 48 to 57% (Table 3.1) and all essential amino acid concentrations (Table 3.3) improved except for tryptophan. However, the protein digestibility of ACCM decreased to 79% (Table 3.8), an effect of high (14%) crude fiber content.

Based on the above observations, a separate batch of hexane extracted CM was subjected to AC to produce (ACCM), which was included at 10 (10% ACCM) and 30% (30% ACCM) of diets containing 10% FM and 10% poultry by-product meal (PBM). We also included 30% of double washed CM that was not aerobically converted (30% WCM) as a treatment diet (Chapter 6). Inclusion of up to 30% of processed CM in diets did not affect fish growth partly because feed intake increased with increasing CM in diets. All levels of ACCM inclusion in diets increased feed conversions and reduced protein efficiency. The condition (Fulton's K) of HSB fed 30% WCM was better than that of HSB fed other diets and feed efficiency was similar to the reference diet. In addition, weight gain of HSB fed 30% WCM ranked highest.

All diets contained similar quantities of FM, PBM and plant meals; and were balanced for crude fiber using carboxymethyl cellulose. Therefore, it was not clear why the second wash in WCM improved fish condition and feed utilization. The most probable reason was higher digestibility of WCM, resulting in release of more nutrients in the blood stream available for metabolic processes. However, the fact that the second wash in WCM concentrated more crude fiber than the single wash in ACCM complicated the notion, requiring follow up studies to determine the physiology behind utilization of ACCM and WCM nutrients in HSB. Therefore, the objectives of the current study were to determine the effect of ACCM and WCM on trypsin activity and consequently, protein digestibility, and amino acid digestibility and bioavailability.

Materials and Methods

After conclusion of the growth trial (Chapter 6), the remaining HSB with an approximate tank average of 80-109g, were maintained in the same 32-tank recirculating

aquaculture system (6-7 replicates/diet) on their respective four diets without chromic oxide (Table 8.1, whole-cleaned wheat replacing chromic oxide). These fish were sacrificed to harvest their pyloric caeca for trypsin activity and to obtain blood and dorsal white muscle to quantify free amino acids. Temperature, pH and dissolved oxygen were monitored daily using a YSI Professional Plus meter (YSI, Yellow Springs, OH) and averaged (mean \pm SD) 25.9 \pm 0.7 $^{\circ}$ C, 7.4 \pm 0.4 and 7.2 \pm 1.1 mg/L, respectively. Unionized ammonia and nitrite were monitored weekly using a Hach DR 2000 spectrophotometer (Hach, Loveland, CO) and averaged (mean \pm SD) 0.013 \pm 0.007 mg/L and 0.26 \pm 0.05 mg/L, respectively.

Harvesting pyloric caeca for trypsin activity assay. —After three weeks of feeding, fish were fasted for 48 hours before harvesting their pyloric caeca. Two randomly selected fish from each tank were euthanized (ms-222) and necropsied to remove their viscera. The viscera were put on an ice block to slow enzyme activity and the fat surrounding the pyloric caeca were removed before excision of the caeca. Caeca were pooled by tank, immediately frozen on dry ice, and later stored at -80 $^{\circ}$ C. Enzyme extracts were prepared as described by Silva et al. (2011). Frozen caeca were weighed, partially thawed on ice, and homogenized at 4mg/mL (w/v) in 0.01M Tris-HCl+ (containing 0.9% NaCl, pH 8.0). Homogenates were centrifuged at 10,000x g in a chilled centrifuge and the collected supernatants were snap frozen on dry ice and later stored at -80 $^{\circ}$ C, pending analysis.

Blood and muscle sampling for free amino acids. —The remaining fish (n~13) in the tanks were fed their respective diets for another three weeks. The fish were then hand graded, so that six fish of similar size were retained in each tank. Individual tank

averages ranged from 173 to 323g. All fish were group weighed and starved for four days. Diets were ground to particles <0.5 mm and mixed with 2X (w/v) water shortly before force-feeding. Fish were force-fed their respective diets by intubation, at a rate of 1.0% of the average fish weight in each tank and the time of force-feeding was recorded. Fish were serially sampled for blood and dorsal white muscle at 0, 3, 6, 9, 12, 24 and 36 hours after force-feeding. One treatment tank was used per sampling interval and pooled samples from three fish provided replicates. All six fish in each tank were euthanized and blood obtained from caudal sever of three fish was pooled into one sample by combining 5-7 drops of blood from each fish into a single centrifuge tube. Centrifuge tubes were immediately put on ice and later kept at 4°C for 12-24 hours before centrifugation. Blood was centrifuged at 1000x g in a chilled centrifuge for 10 mins to collect serum, which was frozen on dry ice and later stored at -80°C. From the same fish, white muscle tissue (~0.5g) was excised from the left side of the dorsal fin, about 2.5 cm anterior to the fin. Muscle tissues from three fish were pooled into one sample in a centrifuge tube, immediately frozen on dry ice and later stored at -80°C.

Serum was deproteinated as described by Yamamoto et al. (2005). Sulfosalicylic acid (10%) was added to an equal volume of serum to precipitate soluble proteins and the mixture was centrifuged at 10,000x g for 10 mins in a chilled centrifuge to collect the supernatant containing free amino acids. The supernatant was frozen at -80°C pending analysis. Muscle free-amino acids were extracted as described by Ogata and Murai (1994). About 100 mg of muscle tissue excised from each of the three muscle samples were pooled into one sample (~300 mg) and homogenized in 3 mL of 0.6N perchloric acid in 15 mL centrifuge tubes using pellet pestles. The homogenates were shaken for 30

mins at 200 rpm, after which they were neutralized with 0.25 mL of KOH (7.2M). The mixture was then centrifuged at 10,000 rpm in a chilled centrifuge for 10 mins and the supernatant containing free amino acids was collected and stored at -80°C , pending analysis.

Protein and amino acid digestibility. —Fifty HSB (350-500g) from a separate batch that had not been fed any experimental diet were separately stocked into each 200-gallon tank of a 5-tank recirculating aquaculture system. Temperature, pH, dissolved oxygen, unionized ammonia and nitrite were monitored as previously described and averaged (mean \pm SD) $26.7\pm0.4^{\circ}\text{C}$, 7.5 ± 0.5 and 6.4 ± 1.3 mg/L, 0.015 ± 0.003 mg/L and 0.108 ± 0.016 mg/L, respectively. Fish were fed a reference diet without a tracer for seven days and then fed test diets (similar to growth trial diets but containing 0.75% chromic oxide, Table 8.1), for seven days, after which abdominal palpation was done to collect distal feces. Fish were combined in one tank after stripping and then randomly separated and restocked in the five tanks and fed a reference diet for another seven days before a new set of test diets were fed. Each test diet was fed to two tanks that functioned as replicates. Stripped feces were frozen and later freeze-dried using a Labconco Freezone 2.5 freeze dryer (Kansas City, MO) for 72 hours.

Freeze-dried feces and diets were finely ground in a coffee grinder and analyzed for crude protein (AOAC 1995, method 972.43) and chromic oxide (Cortes 1979). Amino acids were hydrolyzed from diets and feces using 6M HCl at 110°C for 24 hours, except glutamine and asparagine which are deamidated and tryptophan (Dai et al. 2014), cysteine and methionine (Bunka et al. 2009) that are destroyed. Cysteine and methionine

were pre-oxidized by performic acid to cysteic acid and methionine sulfone, respectively, before acid hydrolysis (AOAC 1995, method 994.12).

Trypsin activity assay. —*N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA, Sigma Cat. No. B4875) dissolved in dimethylsulphoxide was used as a substrate and porcine pancreatic trypsin (Fisher Cat. No. AAJ6399309) was used as a standard. Trypsin activity was measured as described by Silva et al. (2011) on four replicates of duplicate samples. Frozen enzyme extracts were thawed on ice and 140 µL of 0.1M Tris-HCl+ were put in each well of a 96-well microplate. Thirty µL of enzyme extracts, blanks (0.1M Tris-HCl+) or standards were then added, followed by 30 µL of BAPNA (8mM). The plates were incubated at 25°C for 10 mins after which the optical density was read at 405nm using a Bio Tek Epoch plate reader (Winooski, VT). Enzyme activity was reported as µmoles of p-nitroaniline released per gram caeca.

Amino acid analysis. —Cysteic acid (Cat. No. AC226120100, Fisher Scientific, Fair Lawn, NJ) and methionine sulfone (Cat. No. AC439000050, Fisher Scientific, Fair Lawn, NJ) were added to the amino acid standards prepared from the stock solution (Cat No. WAT088122, Waters Corporation, Milford, MA). Free amino acids in samples or standards were derivatized using an AccQ-Tag derivatization kit (Cat. No. 186003836, Waters Corporation, Milford, MA), separated on an AccQ-Tag Ultra C18 1.7µm, 2.1x100 mm column (Cat. No. 186003837, Waters Corporation, Milford, MA) in an Acquity H class UPLC (Waters Corporation, Milford, MA) using reverse-phase chromatography and quantified using an Acquity UPLC PhotoDiode Array (PDA) detector (Waters Corporation, Milford, MA). Amino acid concentrations were reported as pmoles/µL for

deproteinated serum and muscle or as a percent of the hydrolyzed quantity of diets or feces.

Apparent digestibility coefficient (ADC, %) of protein and amino acids. —ADCs of protein or amino acids were calculated using the equation (NRC 2011):

$$\text{ADC}_{\text{protein/amino acids}} = 1 - \frac{\text{Cr}_2\text{O}_3 \text{ in feed}}{\text{Cr}_2\text{O}_3 \text{ in feces}} \times \frac{\text{protein/amino acid content in feces}}{\text{protein/amino acid content in feed}}$$

Statistical analysis—All statistical analysis was completed with version 3.4.3 of R statistical software (R Core Team 2017). Non-normal data distributions were power transformed before statistical analysis. Trypsin activity and digestibility data were analyzed by One-Way Analysis of Variance (ANOVA) and Tukey's HSD test used to identify treatment differences. All serum and muscle free amino acid temporal data were analyzed using repeated measures ANOVA except for muscle tryptophan that was analyzed using Friedman's test. Least square means were used for multiple comparisons. Treatment means were considered different at $P < 0.05$.

Results

Trypsin activity, protein and amino acid digestibility. —Inclusion of up to 30% ACCM or WCM in diets did not change ($p=0.586$) intestinal trypsin activity (Table 8.2). Although there was no statistical difference ($p=0.112$) in protein ADCs with processed CM inclusion in diets (Table 8.2), protein ADCs of ACCM diets ranked lower than those of the reference and 30% WCM diets. Inclusion of processed CM did not alter the ADC of arginine, histidine, lysine, methionine or cysteine and tyrosine (Table 8.2). The ADCs of isoleucine, leucine, phenylalanine and valine for the 30% ACCM diet were lower than those other diets. The ADC of threonine for the 30% WCM, the reference and 10%

ACCM diets were similar but higher than that of the 30% WCM diet. The ADC of threonine for the reference and ACCM diets were similar.

Serum free amino acids. —Lysine, threonine and cysteine concentrations did not vary with inclusion of processed CM in diets or with sampling time after force-feeding (Table 8.3). Increasing ACCM reduced arginine concentrations. Initial arginine concentrations were high for all diets and reduced to the lowest concentration at 3 hours and then gradually increased, peaking at 12 hours and remained high until 36 hours. There was no effect of processed CM on histidine concentrations. Initial histidine concentrations were high but decreased to lowest concentrations at 3 hours and then gradually increased, peaking at 9 hours and then reduced after 12 hours. There was no effect of processed CM on isoleucine concentrations. Initial isoleucine concentrations were high and decreased to the lowest concentration at 6 hours and then gradually increasing, peaking at 36 hours. Leucine concentrations of HSB fed the reference, 10% ACCM and 30% WCM were similar but higher than that of HSB fed 30% ACCM. Leucine concentrations of HSB fed 30% ACCM and 30% WCM were similar. Leucine concentrations peaked at 3 hours and then gradually decreased, reaching the lowest concentrations at 24 hours and remained low at 36 hours. Inclusion of processed CM in HSB diets increased methionine concentrations but had no effect on serum phenylalanine concentrations. However, both methionine and phenylalanine concentrations increased at 3 hours and remained high until 9 hours, after which they started declining, reaching the lowest concentrations at 24 hours. Tryptophan concentrations of RBT fed 30% ACCM or WCM were similar and higher than those of RBT fed the reference and 10% ACCM diets. The tryptophan concentration of RBT fed the reference diet was higher than that of

RBT fed 10% ACCM. Initial tryptophan concentrations were high and remained high until 12 hours, after which they reduced to lowest concentrations. RBT fed 30% WCM had similar valine concentrations to RBT fed the reference and 30% ACCM diets but higher than that of RBT fed 10% ACCM. RBT fed the reference, 10% ACCM and 30% ACCM had similar valine concentrations. Initial valine concentrations were high and reduced gradually to lowest concentrations at 6 and 9 hours and did not significantly rise after 9 hours. There was no effect of inclusion of processed CM on tyrosine concentrations. Tyrosine concentrations gradually increased, peaking at 6 hours and then gradually reduced to the lowest concentration at 24 and 36 hours.

Muscle free amino acids. —Inclusion of processed CM did not alter arginine concentrations. Arginine concentrations were similar up to 24 hours and peaked at 36 hours. Higher inclusions of processed CM increased histidine concentrations, with HSB fed 30% ACCM having more histidine than HSB fed 30% WCM. Histidine concentrations gradually increased, peaking at 6 hours and remained high after 6 hours. There was no effect of inclusion of processed CM on isoleucine concentrations. Initial isoleucine concentrations were high and reduced to the lowest concentrations at 3 hours and then gradually increased, peaking at 36 hours. Inclusion of 30% ACCM in diets of HSB reduced leucine concentrations. Initial leucine concentrations were higher but reduced to lowest concentrations at 3 hours and gradually increased, peaking at 9 hours and never reduced after 9 hours. Inclusion of 30% WCM in HSB diets increased lysine concentrations but there was no sampling time effect on lysine concentrations. Inclusion of processed CM increased methionine concentrations. Methionine concentrations gradually increased, peaking at 6 hours and remained high until 12 hours, after which

they reduced to lowest concentrations at 24 and 36 hours. Phenylalanine concentrations reduced with increasing processed CM in HSB diets. Phenylalanine concentrations gradually increased, peaking at 9 hours and then gradually reduced to the lowest concentration at 24 hours. There was no diet or sampling time effect on threonine concentrations. There was no effect of processed CM inclusions in HSB diets on tryptophan concentrations. Inclusion of 30% WCM in HSB diets increased valine concentrations. Initial valine concentrations were high but reduced to the lowest concentration at 3 hours and then gradually increased, peaking at 24 and 36 hours. Cysteine was detected only in HSB fed a diet containing 10% ACCM at 9 hours. Inclusion of 30% ACCM in HSB diets reduced tyrosine concentrations. Initial tyrosine concentrations were high but reduced to lowest concentrations at 3 hours and then gradually increased, peaking at 9 hours and remained high after 9 hours.

Discussion

Trypsin activity, protein and amino acid digestibility.—Inclusion of up to 30% of processed CM did not alter trypsin activity because carinata contains very low concentrations of trypsin inhibitors (≤ 2.4 TIU/g, Bell and Rakow 1996). In addition, raw wheat and corn contain 4.7 and 7.5 mg of trypsin inhibitors/g of meal, respectively (Venou et al. 2003). The tolerance limit of trypsin inhibitors is not documented for HSB but could be similar to that of Rainbow Trout *Oncorhynchus mykiss* (RBT). It requires inclusions of $\geq 64\%$ of either raw wheat or corn to contribute enough trypsin inhibitors to reach the tolerance limit of 5 mg of TIU/g of diet reported for RBT (Krogdahl et al. 1994). Therefore, it would require that HSB to be more sensitive to trypsin inhibitors than RBT, for raw wheat and corn to have any significant effect on trypsin activity. Isolation

of starch to concentrate proteins in wheat gluten (Day et al. 2006) and possibly corn protein concentrate (CPC, Emphyreal75) may concentrate trypsin inhibitors in such meals. However, considering the very low initial concentrations of trypsin inhibitors in wheat and corn and their potential degradation during processing into higher protein meals, varying concentrations of wheat gluten and CPC had no effect on trypsin activity.

Diets were cook extruded and the heat involved in extrusion (Barrows et al. 2007) and drying of diets would have resulted in reduction of trypsin inhibitors. Diet extrusion would not have completely inactivated high trypsin inhibitors without prior reduction of trypsin inhibitors by ingredient processing, if diets contained ingredients such as raw soybean (62,081 TIU/g, Pisulewska and Pisulewska 2000; 43-84,000 TIU/g, Guillaumon et al. 2008), peas (3,673 TIU/g, Pisulewska and Pisulewska 2000) and camelina (10,800-13,400 TIU/g, Almeida et al. 2013). Processing such ingredients requires roasting/heating to temperatures of about 100⁰ C for a minimum of 30 minutes (Jourdan et al. 2007) to inactivate their high trypsin inhibitors before incorporation into fish diets. Feeds in the current study were extruded at 96⁰ C, with a residence time of 3 mins but could reach 150⁰ C in some extrusions (Rokey et al. 2010). Residence times of feed in the extruder barrel are usually short. Therefore, residence times (18-37 seconds) as the time in the current study or in the study by Barrows et al. (2007) are insufficient considering the 30 minutes required to inactivate all trypsin inhibitors in the study by Jourdan et al. (2007). The drying temperature for the feed was 101⁰ C, which is between 90 and 180⁰ C reportedly used to dry feed (Rokey et al. 2010) but the drying time of about 6.5 mins may not be long enough for complete trypsin inhibitor inactivation.

Phytic acid was reported to reduce trypsin activity *in-vitro* (Singh and Krikorian 1982) and in sows (Mroz et al. 1995). Processed de-oiled CM involving a single wash contained 3.1% phytic acid (Table 3.5). Whole wheat and corn contain <1% phytate (Kumar et al. 2012). Residual phytate in wheat middlings are likely more than those in wheat gluten and CPC but may be lower than the phytate concentration of ACCM. Therefore, the impact of phytates on trypsin activity would increase with increasing ACCM or WCM in diets. However, trypsin activity did not differ among HSB fed up to 30% ACCM or WCM. Trypsin activity of HSB fed 30% WCM ranked higher possibly because WCM was not aerobically converted and may have contained more phytates than ACCM that was converted with fungi. Dietary phytates in the current study were not analyzed. However, diets of Atlantic Salmon *Salmo salar* containing up to 1.0% phytates did not alter trypsin activity (Sajjadi and carter 2004).

Protein ADCs did not change with increasing processed CM in diets. However, the ranking of protein ADCs of diets containing ACCM decreased with increasing ACCM content in diets, likely due to the low protein ADC of ACCM (79%, Table 3.8). The low protein ADC of ACCM was because diets were not cook-extruded. However, even with a correction factor of 5.8 applied to ACCM after comparing the protein ADCs of the cold pressed reference diet in the current study and the cook-extruded counterpart in Barrows et al. (2015), the protein ADC of ACCM would still be lower (84.8%) than those of most ingredients used in the diets. Inclusions of wheat gluten, whole wheat and CPC decreased with increasing ACCM in diets. This resulted in reduction of dietary protein ADC because wheat gluten (100%), whole wheat (92%) and CPC (88%) have higher protein ADCs (Barrows et al. 2015) than ACCM. Although the protein ADC of

whole corn (100%, Barrows et al. 2015) is higher than that of ACCM, corn did not vary (9.4-9.85%) among the reference and ACCM diets. The low protein ADC (50%) of wheat middlings (Barrows et al. 2015) had less effect on the protein ADC of ACCM diets because its concentrations reduced with increasing ACCM in diets.

Concentrations of CPC, corn, and wheat gluten were lowest in the diet containing 30% WCM and the concentration of whole wheat was lower than those of the reference and 10% ACCM diets but close to that of 30% ACCM diet. However, the protein ADC of 30% WCM diet ranked higher than those of other diets did, which suggests a higher protein ADC of WCM than ACCM.

High inclusion of ACCM reduced isoleucine, leucine, phenylalanine, threonine and valine ADCs because of the potentially low ADCs of these amino acids in ACCM. This is supported by the high (>90%) ADCs of isoleucine, leucine, phenylalanine, threonine and valine in wheat gluten, whole wheat, corn and wheat middlings (Barrows et al. 2015). The ADCs of similar amino acids are lower (84 to 93%) in CPC. However, the 30% ACCM diet had a lower concentration of CPC than the reference and 10% ACCM diets.

The ADCs of all EAAs were higher or close to their dietary protein ADCs except for methionine ADCs for 30% ACCM and 30% WCM diets that were lower than their protein ADCs. Crude protein measures the total nitrogen content of a sample (AOAC 2006, method 972.43) and compounds such as nucleic acids, phospholipids, urea, and ammonia contribute to the total nitrogen content of a sample in addition to amino acids (Mariotti et al. 2008). The ADCs of such compounds could account for ADC differences between crude protein and EAAs.

Serum free amino acids. —Isoleucine concentrations associated with dietary isoleucine ADC. The low ADC of valine for the 30% ACCM diet did not lower valine concentrations possibly because the high dietary valine concentration (Table 6.2) could have compensated for the low valine ADC. Reduction in ADCs of isoleucine, phenylalanine and threonine did not lower the concentrations of these amino acids in serum. However, methionine concentrations in serum increased with all processed CM inclusions in diets, although, dietary methionine ADC did increase with increasing processed CM.

Differences in amino acid ADCs may not have been reflected in serum because of individual variation in fish sampled by serial slaughter (Ok et al. 2001). Pooling blood from many fish into a single sample may reduce variation among individual fish. However, unrepresentative quantities of blood by fish weight, quantity of diet fed, etc., contributed by different fish reduce the efficacy of pooled samples.

Digesta movement in the gastrointestinal tract of fasted HSB was a minimum of 8 hours. However, amino acid absorption started before a minimum of 3 hours and lasted the entire 36 hours for some amino acids. Therefore, most dietary effects on amino acid ADCs would be reflected in blood if the liver or extra-hepatic tissues never simultaneously drew amino acids from the blood stream for metabolism.

Blood was sampled post-liver from the caudal vasculature and the quantity of amino acids in hepatic portal vein removed by the liver on the first pass is unknown. However, estimates could be as high (Karlsson et al. 2006) as those (20-50%) in humans (Hoerr et al. 1991; Biolo et al. 1992; Hoerr et al. 1993; Matthews et al. 1993). Blood sampled pre-liver in the hepatic portal vein would have shown a better association of

dietary ADCs with serum amino acids (Karlsson et al. 2006). However, we did not sample blood from hepatic portal vein due to the small size of fish.

Amino acid concentrations in blood from arteries are typically higher than in blood from the caudal vasculature (Ok et al. 2001). This is because blood from the caudal vasculature is a combination of arterial and venous blood. Therefore, dietary protein ADCs were not reflected in serum possibly because of the dilution of arterial by venous blood in the sampled blood. However, dilution of arterial by venous blood is not high enough to change the pattern of amino acid absorption over time. Ok et al. (2001) observed similar amino acid patterns over time for both dorsal aorta cannulated and serially slaughtered fish. However, stress from repeated sampling in cannulated fish may have led to increased proteolysis, increasing plasma free amino acids (Vander Boon et al. 1991; Vijayan et al. 1991) and thus a generally higher concentration of amino acids in dorsal aorta blood than the combined arterial and venous blood in the caudal vasculature.

The peak times of individual amino acids in the current study varied more than those of similar amino acids in RBT (Table 7.3) yet test diets in the RBT study contained more plant meals. This may explain the poor feed utilization and thus growth rates of HSB (Table 6.3) as opposed to RBT (Table 5.3). Crystalline amino acids are absorbed faster than amino acids in intact proteins (Schuhmacher et al. 1997; Rodehutscord et al. 2000). All diets used in the current study were supplemented with lysine (0.1-0.2%), methionine (0.2%) and tryptophan (0.12%). There was no effect of lysine supplementation on lysine patterns over time, which could be because of the small quantities of supplemented lysine. However, supplementation of similar quantities of methionine resulted in an early peak (3 hours) of methionine. High early (3-12 hours)

peaks of tryptophan may not be fully attributed to supplemented tryptophan because initial (0 hours) tryptophan concentrations were high and the quantity of supplemented tryptophan was lower than that of lysine and methionine.

Crystalline amino acids in RBT diets in the study by Ok et al. (2001) resulted in amino acid absorption peaks at 4 hours after force-feeding. Intact amino acids in the study by Karlsson et al. (2006) peaked earlier (6 hours) than in the current study because diets contained a higher (34-51%) percentage of FM. However, when Schuhmacher et al. (1997) force-fed 100% wheat gluten to RBT, amino acids peaked later (18 hours) than in the current study where diets contained 63-65% of a mixture of plant meals. Most EAA and CEAA (conditional) in the current study peaked between 3 and 12 hours, with most EAAs having extended peaks. However, they returned to baseline levels at 24 hours after force-feeding except for arginine and isoleucine that peaked at 9 and 12 hours, respectively and never returned to baseline levels. Larsen et al. (2012) replaced 59% of FM with a mixture of plant meals and observed most EAAs peaking between 6 and 12 hours. However, more than half of the EAAs reached baseline levels after 24 hours. Generally, amino acids from intact proteins are released into the blood stream slower (Tantikitti and March 1995) than crystalline amino acids. However, the slower release of amino acids with FM replacement by plant meals suggests a slower release of amino acids from plant than animal meals.

Total EAAs in fish fed processed CM diets slightly increased or decreased (Figure 8.1) 3 hours after force-feeding compared total EAAs in fish fed the reference diet; Lyndon et al. (1993) made a similar observation. All fish in the current study were similarly handled, anesthetized and force-fed. Therefore, delay or slow release of EAA

from diets containing processed CM could be attributed to the slow release of EAA from processed CM and not reduced intestinal flow due to handling stress and anesthesia (Thorarensen et al. 1993; Eliason et al. 2007) or delayed gastric emptying due to force-feeding (Dos Santos and Jobling 1988). The high increase in EAAs at 3 hours followed by a high reduction in EAAs at 6 hours in fish fed the reference diet cannot be fully attributed to increase in muscle EAAs because it was low. Therefore, this may suggest increased EAA metabolism in the liver at 6 hours.

The reference diet resulted in two peaks that were higher than those of the ACCM diets were but lower than that of the 30% WCM diet. Increasing ACCM (30%) in the diet led to two peaks compared to a single but relatively broader peak observed in fish fed less (10%) ACCM. Inclusion of WCM in the diet resulted in a single but higher and broader peak. This suggest that high concentrations of EAAs from WCM are released over a longer period. The two peaks observed in fish fed the reference and 30% ACCM may not be attributed to other ingredient inclusion levels in the diets because peaks were lower in 30% than 10% ACCM. The gradients of cumulative total EAAs (Figure 8.2) for fish fed ACCM diets were surpassed after 6 hours by that of fish fed 30% WCM. The gradient of fish fed 30% WCM surpassed that of fish fed the reference diet after 12 hours and the difference increased at 24 and 36 hours.

Muscle free amino acids. —Increase in histidine concentrations with increasing processed CM in diets (Table 8.4) cannot be explained by histidine concentrations in serum because they did not differ with dietary treatment. Initial concentrations of histidine in HSB fed 30% processed CM were much higher than for HSB fed the reference and 10 ACCM diets. In addition, the initial concentration of histidine for HSB

fed 30% ACCM was higher than that of HSB fed 30% WCM. This initial pattern of histidine concentration was maintained throughout the 36-hour sampling period. Leucine concentrations associated with serum leucine concentrations. Increase in lysine concentration in HSB fed 30% WCM cannot be explained by lysine concentrations in serum because they did not differ with dietary treatment. The higher concentration of lysine in HSB fed 30% WCM than in HSB fed other diets may explain observed differences in growth (Table 6.3) because of the high lysine requirement for growth based on muscle lysine concentrations relative to other EAAs (Rawles et al. 2012). Methionine concentrations associated with serum methionine concentrations. Reduction in phenylalanine and tyrosine concentrations with increasing processed CM in diets cannot be explained by phenylalanine concentrations in serum because they did not differ with dietary treatment.

Patterns of total EAAs in muscle did not fully associate with those of serum (Figure 8.1) because amino acids' entry into muscle cells is not passive (Lyndon et al. 1993) based on concentration gradients. The active nature of amino acid movement into cells enables maintenance of high amino acid concentrations in cells even in states of low supply by blood. Patterns of total EAAs of HSB fed the reference and 30% WCM diets associated with those of serum better than those of HSB fed ACCM diets. Total EAAs in HSB fed the reference and 30% WCM increased at 3 and 6 hours. However, total EAAs in ACCM diets decreased at 3 hours and total EAA of HSB fed 10% ACCM diet decreased further at 6 hours even with increase in serum total EAAs at 6 hours. The slight reduction in total EAAs at 9 hours for HSB fed 30% WCM cannot be explained by total EAAs in serum because it increased from 6 to 9 hours. Reduction of total EAAs at 24

hours for HSB fed the reference and 30% ACCM may be attributed to the low concentrations of total EAAs in serum at 24 hours. However, the low concentration of total EAAs in serum at 24 hours for HSB fed 10% ACCM did not reduce the concentration of total EAAs at 24 hours. Highest processed CM resulted in broader peaks of total EAAs which may explain why 30% ACCM did not reduce fish growth (Table 6.3) despite ranking lowest in dietary protein ADC.

No dietary EAA ADC differences were reflected in muscles except for leucine. No studies have determined the association of dietary amino acid ADCs with muscle free amino acids. However, Lyndon et al. (1993), Carter et al. (1995) and Yamamoto et al. (2000) observed a poor association of dietary amino acids with muscle free amino acids. This could be attributed to the contribution of amino acids from protein catabolism to the total free amino acid pools accrued in muscle cells (Cowey and Walton 1989).

The composition of EAAs in intact proteins does not change in a given species or strain of fish with diet. Based on this notion, we used all fillet EAA concentrations in the study by Rawles et al. (2012) to calculate EAA to lysine ratios, except for the tryptophan to lysine ratio that was calculated based on EAA concentrations reported in USDA (1987). Rawles et al. (2012) did not analyze tryptophan. We used calculated EAA to lysine ratios to calculate concentrations of EAAs expected to be available for muscle protein synthesis at a given sampling time. Then we divided the actual concentrations of EAAs by the expected concentrations at each sampling time to determine if a given EAA was limiting (<1.0) or adequate (>1.0) for protein synthesis (Table 8.5). However, we did not account for what is required for maintenance.

We observed that only histidine concentrations were adequate for muscle protein synthesis throughout the 36-hour sampling period (Table 8.5). Unlike in RBT (Table 7.5), tryptophan in HSB was less limiting to protein synthesis than arginine and isoleucine. Tryptophan was also available for protein synthesis after 3 hours except in the RBT study (Table 7.5) where it was only available at 0 and 36 or more hours. Very low tryptophan was detected in the muscle of Atlantic Cod *Gadus morhua* fed Sand Eels *Ammodytes spp.* (Lyndon et al. 1993). Yamamoto et al. (2000) did not detect tryptophan in muscles of RBT fed different protein concentrations. Phenylalanine was found to be limiting to protein synthesis in addition to tryptophan in Atlantic Salmon *Salmo salar* fed a low ration (Carter et al. (2000). All the above studies based their conclusions on muscle concentrations not concentrations relative to empirical requirements. Because EAA requirements differ, the limitation for protein synthesis should be based on concentrations relative to requirement as calculated in Table 8.5.

No sampling time was optimal for protein synthesis because all EAAs except histidine concentrations were inadequate. In the RBT study (Table 7.5), optimal time for protein synthesis occurred at 36 or more hours after force-feeding because all EAA concentrations were adequate. This may explain better growth rates and feed utilization in RBT (Table 5.3) than in HSB (Table 6.3). We did not observe an elevation in a single EAA as did occur in RBT (Table 7.4), resulting in an EAA to lysine ratios of up to 320 for histidine (Table 7.5). Although the EAA to lysine ratios were also highest for histidine in the current study, they were much lower (≤ 4.5). We did not observe any dietary effect of processed CM on EAA to lysine ratios. Cumulative EAAs increased with

increasing processed CM in diets (Figure 8.3). Cumulative EAAs of HSB fed 30% WCM surpassed those of 30% ACCM after 12 hours.

Conclusion

Double washing CM improved its utilization by increasing apparent protein and EAA digestibility. However, it is not clear why the second wash improves digestibility because it slightly concentrates more crude fiber from 10.2% in the first wash to 11.0%. The high methionine content of processed CM resulted in high serum methionine contents, which shows benefits of using CM as opposed to other plant meals because methionine is always among the most limiting EAAs when a large percentage of plant meals is included in fish diets. Ratios of EAAs to lysine showed that more processed plant meals in addition to low-cost, animal by-product meals such as blood meal, chicken by-product meal, etc., should be included in HSB diets to improve the concentrations of available EAAs. Processing of CM should improve amino acid composition and reduce the release time of amino acids into the blood stream by reducing fiber and possibly reducing the length of peptides in CM, thus improving the potential of processed CM to replace FM or other animal meals fish diets.

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Table 8.1. Formulation (g/100g, dry basis) of diets used for protein and amino acid digestibility in Hybrid Striped Bass^a.

Ingredients	Reference	Carinata		
		10 % ACCM ^a	30 % ACCM ^a	30% WCM ^a
Fish meal (special select) ^b	10.00	10.00	10.00	10.00
ACCM ^c	0.00	10.00	30.00	0.00
WCM ^c	0.00	0.00	0.00	30.00
Fermented soy-Gen1 Batch#3 ^c	10.00	10.00	10.00	10.00
Poultry by-product meal ^d	10.00	10.00	10.00	10.00
Empyreal 75 ^e	9.80	9.00	3.20	1.30
Wheat Midds ^f	10.00	6.96	4.63	5.18
Corn ^g	9.70	9.85	9.40	9.00
Whole-cleaned wheat ^g	13.33	11.27	5.45	7.85
Wheat gluten ^h	10.00	6.30	2.00	1.50
CMC ⁱ	2.60	1.85	0.10	0.00
Vitamin premix ^f	1.50	1.50	1.50	1.50
Mineral premix ^f	1.50	1.50	1.50	1.50
Stay C ^j	0.30	0.30	0.30	0.30
Choline (60%) chloride ^k	0.30	0.30	0.30	0.30
Lysine ^l	0.20	0.20	0.20	0.10
Tryptophan ^l	0.12	0.12	0.12	0.12
Methionine ^m	0.20	0.20	0.20	0.20
Mono calcium phosphate ⁿ	1.00	1.00	1.00	1.00
Menhaden, fish oil ^b	8.00	8.00	8.00	8.00
Soybean oil ^o	0.70	0.90	1.35	1.40
Chromic oxide ^p	0.75	0.75	0.75	0.75
Proximate composition				
Crude protein (%) ^q	43.6	44.0	44.9	45.3
Crude lipid (%) ^r	12.1	12.1	12.	12.1
Crude fiber (%) ^r	5.5	5.4	5.5	5.5
Ash (%) ^r	8.6	9.0	10.1	10.2
NFE (%) ^r	24.6	25.0	24.1	25.5
Gross energy (GE, MJ/Kgr ^r	19.89	20.07	20.21	20.05
Protein:Energy (g/MJ) ^r	21.15	21.52	21.70	21.02
Glucosinolates (μmolesg ⁻¹) ^q	0.00	0.042	0.51	0.54

^aDiets formulated to contain 42% crude protein and 12% crude lipid. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC; ^bOmega proteins, Houston, TX; ^cSouth Dakota State university, Brookings, SD; ^dTyson Foods Inc., Springdale, AR; ^eCargill, Blair, NE; ^fNutra Blend LLC, Neosho, MO; ^gAg First Farmers Co-op, Brookings, SD; ^hManildra Milling Corporation, Leawood, KS; ⁱAkzo Nobel Functional Chemicals BV, Amersfoort, Netherlands; ^jDSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China; ^kBiochem Corporation, New Hampton, NY; ^lAnjinomoto Animal Nutrition group, Chicago, IL; ^mAdiddeo Blue Star Company, North Point Parkway, GA; ⁿPCS sales, Northbrook, IL; ^oSouth Dakota Soybean Processors, Volga, SD; ^pFisher Scientific, Pittsburg, PA; ^qAnalyzed; ^rCalculated.

Table 8.2. Effects of processed carinata meal on trypsin activity (TA), apparent digestibility coefficients (ADCs, %) of protein, essential (EAA) without tryptophan and conditionally (CEAA) essential amino Acids in Hybrid Striped Bass^a.

Variable ^a	Reference ^a	10% ACCM ^a	30% ACCM ^a	30% WCM ^a	p-values
TA (U/mg of tissue) ^b	11.6±1.3	10.1±1.4	11.1±2.1	14.0±2.9	0.586
ADCs (%)					
EAA					
Protein	72.8±2.3	69.7±0.2	59.0±3.9	73.6±3.4	0.112
Arginine	80.3±5.0	78.9±0.8	86.6±13.4	87.9±1.3	0.766
Histidine	73.6±6.8	75.4±0.6	58.0±7.4	83.3±2.5	0.102
Isoleucine	87.0±2.8 ^a	81.8±1.2 ^a	68.8±2.0 ^b	87.8±1.4 ^a	0.007
Leucine	90.8±1.7 ^a	85.1±1.3 ^a	70.9±0.0 ^b	86.9±0.3 ^a	0.001
Lysine	97.0±0.2	91.4±2.0	100.0±0.0	95.8±4.2	0.220
Methionine	77.4±3.0	75.7±1.9	52.2±0.3	65.6±10.7	0.098
Phenylalanine	84.3±3.6 ^a	80.3±0.7 ^a	61.9±4.6 ^b	85.2±1.4 ^a	0.016
Threonine	78.1±5.2 ^{ab}	74.9±1.4 ^{ab}	59.2±4.7 ^b	81.6±1.0 ^a	0.040
Valine	85.6±3.1 ^a	80.0±1.2 ^a	66.7±3.1 ^b	85.9±0.3 ^a	0.011
CEAA					
Cysteine	22.4±6.8	25.1±9.1	34.0±2.5	54.5±8.2	0.102
Tyrosine	76.6±6.0	77.8±1.1	57.0±8.7	85.1±2.7	0.083

^aDiets formulated to contain 42% crude protein and 12% crude lipid. All diets contained 10% fish meal (FM) and 0% poultry-by-product meal. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC; ^bμmoles of p-nitroaniline released from *N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride per mg of pyloric caeca.

Table 8.3. Effect of processed carinata meal on serum free essential (EAA) and conditionally essential (CEAA) amino acids (nmol/mL) in Hybrid Striped Bass^a.

Diet	EAA	Sampling times (hours)							p-values		
		0 ^{abc}	3 ^c	6 ^{bc}	9 ^{ab}	12 ^a	24 ^{abc}	36 ^a	Diet	Time	Interaction
Reference ^a	Arg	629±12	620±132	546±5	624±75	690±35	543±26	652±25	0.0008	0.0004	0.1553
10% ACCM ^{ab}		505±67	468±33	535±37	632±45	590±4	561±35	643±13			
30% ACCM ^b		534±15	463±28	481±39	473±11	555±35	518±15	573±24			
30% WCM ^a		547±31	420±7	524±34	662±9	676±26	639±2	580±45			
Diet		0^{ab}	3^d	6^{bc}	9^a	12^{ab}	24^c	36^c			
Reference	His	151±11	100±15	112±5	141±10	128±11	118±1	114±2	0.1245	<0.0001	0.2451
10% ACCM		129±11	90±6	118±9	143±1	132±1	119±2	121±5			
30% ACCM		136±0	103±5	120±4	134±2	136±5	119±2	123±4			
30% WCM		136±12	107±7	1425	146±11	152±9	122±0	109±3			
Diet		0^{ab}	3^{abc}	6^c	9^{bc}	12^{abc}	24^{abc}	36^a			
Reference	Ile	151±5	192±47	114±4	122±8	124±13	126±0	157±11	0.1299	0.0033	0.1041
10% ACCM		162±11	126±9	114±4	121±17	113±8	121±5	170±7			
30% ACCM		147±2	137±27	127±13	119±3	147±1	121±5	146±1			
30% WCM		151±12	133±4	136±5	142±4	152±1	164±1	160±21			
Diet		0^{bc}	3^a	6^{ab}	9^{ab}	12^{ab}	24^c	36^c			
Reference ^a	Leu	252±9 ^{bc}	429±97 ^a	294±1 ^{abc}	309±17 ^{ab}	300±24 ^{abc}	227±2 ^{bc}	258±19 ^{abc}	0.0033	0.0003	0.041
10% ACCM ^a		280±16 ^{abc}	300±15 ^{abc}	284±10 ^{abc}	304±36 ^{abc}	272±7 ^{abc}	218±12 ^{bc}	277±13 ^{abc}			
30% ACCM ^b		252±7 ^{bc}	282±40 ^{abc}	258±22 ^{bc}	233±8 ^{bc}	271±5 ^{abc}	207±8 ^c	240±0 ^{bc}			
30% WCM ^{ab}		258±18 ^{bc}	261±2 ^{bc}	258±9 ^{bc}	268±3 ^{abc}	287±5 ^{abc}	285±2 ^{abc}	273±35 ^{abc}			
Diet		0	3	6	9	12	24	36			
Reference	Lys	183±28	177±64	142±11	149±10	141±16	120±4	168±14	0.6444	0.2544	0.3955
10% ACCM		183±8	120±15	146±20	139±24	119±24	126±0	185±17			
30% ACCM		178±15	174±36	161±20	126±1	155±3	142±15	161±1			
30% WCM		143±8	143±4	154±5	154±29	150±16	165±3	117±8			
Diet		0^c	3^a	6^a	9^{ab}	12^b	24^c	36^c			
Reference ^b	Met	101±2	163±20	154±6	141±9	125±11	78±2	72±4	<0.0001	<0.0001	0.5108
10% ACCM ^a		98±1	163±7	176±8	158±10	143±14	91±0	98±1			
30% ACCM ^a		122±1	173±7	185±5	164±6	142±2	109±2	99±2			
30% WCM ^a		115±9	156±13	174±4	165±15	162±6	111±4	104±4			
Diet		0^c	3^a	6^a	9^{ab}	12^b	24^c	36^c			
Reference	Phe	118±6	182±17	171±7	164±6	138±5	88±3	90±1	0.6151	<0.0001	0.2897
10% ACCM		105±1	159±16	173±12	167±7	144±8	90±1	102±2			
30% ACCM		121±1	156±0	175±11	154±6	150±4	101±0	108±8			
30% WCM		119±5	159±1	183±8	153±12	148±9	113±0	105±4			

Table 8.3. Continued

Diet	EAA	Sampling times (hours)							p-values		
		0	3	6	9	12	24	36	Diet	Time	Interaction
Reference	Thr	131±19	132±46	103±7	293±139	299±158	94±8	128±5	0.0780	0.0667	0.4640
10% ACCM		130±8	88±12	131±23	142±12	130±13	121±5	138±2			
30% ACCM		145±0	239±152	264±170	103±2	243±122	108±8	151±8			
30% WCM		148±13	105±16	258±112	304±165	357±164	387±174	149±9			
		0^{ab}	3^{ab}	6^a	9^a	12^a	24^c	36^{bc}			
Reference ^b	Trp	28±0	29±2	29±1	29±0	28±0	24±1	26±1	<0.0001	<0.0001	0.2242
10% ACCM ^c		25±0	25±2	28±0	27±1	27±1	23±1	26±0			
30% ACCM ^a		32±1	28±1	31±1	31±1	32±2	26±0	27±1			
30% WCM ^a		29±1	30±1	32±1	29±2	31±1	25±0	27±1			
		0^a	3^{ab}	6^b	9^b	12^{ab}	24^{ab}	36^{ab}			
Reference ^{ab}	Val	331±12	409±88	272±4	278±19	284±25	271±7	318±23	0.0385	0.0087	0.0859
10% ACCM ^b		365±16	307±19	271±15	280±37	268±16	273±7	342±16			
30% ACCM ^{ab}		338±12	316±36	298±20	280±8	331±1	277±4	308±4			
30% WCM ^a		333±20	316±6	319±15	323±11	346±10	371±3	33632			
	CEAA	0	3	6	9	12	24	36			
Reference	Cys	6±0	7±2	7±1	6±1	7±0	5±0	5±0	0.0967	0.1254	0.0571
10% ACCM		6±0	5±2	6±0	8±1	7±0	5±0	7±0			
30% ACCM		7±1	5±0	6±1	6±0	7±1	5±0	6±1			
30% WCM		6±0	6±0	7±1	6±1	11±5	18±0	6±1			
		0^b	3^{ab}	6^a	9^{ab}	12^{ab}	24^c	36^c			
Reference	Tyr	81±0	96±9	97±4	97±1	86±10	51±2	50±1	0.3130	<0.0001	0.1191
10% ACCM		66±3	73±13	101±1	85±9	78±7	53±2	65±3			
30% ACCM		79±0	80±1	90±2	79±3	87±6	59±1	62±3			
30% WCM		78±7	83±8	91±7	82±6	88±1	60±0	61±5			

^aDiets formulated to contain 42% crude protein and 12% crude lipid. All diets contained 10% fish meal (FM) and 0% poultry-by-product meal. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC.

Table 8.4. Effect of processed carinata meal on muscle free essential (EAA) and conditionally essential (CEAA) amino acids (nmoles/mL) in Hybrid Striped Bass^a.

Diet	EAA	Sampling time (hours)							p-values		
		0 ^{ab}	3 ^b	6 ^b	9 ^b	12 ^{ab}	24 ^{ab}	36 ^a	Diet	Time	Interaction
Reference	Arg	70±3	75±12	59±12	91±14	126±25	92±2	135±6	0.1872	0.0016	0.0002
10% ACCM		233±72	99±7	61±13	48±13	64±21	79±3	213±28			
30% ACCM		115±16	102±7	114±47	103±22	114±13	96±2	120±1			
30% WCM		76±5	105±22	113±14	90±1	137±21	158±1	106±1			
		0 ^b	3 ^{ab}	6 ^a	9 ^{ab}	12 ^{ab}	24 ^{ab}	36 ^{ab}			
Reference ^c	His	940±271	1086±35	1343±45	1251±149	1046±146	1151±75	1263±161	<0.0001	0.0403	0.6335
10% ACCM ^c		983±237	1106±63	1417±59	1410±34	1507±97	1371±90	1126±121			
30% ACCM ^a		1819±217	2198±130	2103±113	2111±5	2423±146	2028±117	1891±202			
30% WCM ^b		1590±72	1566±125	1688±198	1552±85	1683±265	1469±4	1525±124			
		0 ^a	3 ^e	6 ^{de}	9 ^{de}	12 ^{cd}	24 ^{bc}	36 ^{ab}			
Reference	Ile	123±8	49±2	51±6	84±3	53±6	105±4	120±16	0.0771	<0.0001	0.0924
10% ACCM		145±14	39±0	54±22	52±3	70±8	106±3	102±13			
30% ACCM		165±47	41±2	80±2	74±4	97±3	86±4	115±1			
30% WCM		111±4	63±3	88±7	72±15	98±0	128±3	131±23			
		0 ^a	3 ^b	6 ^{ab}	9 ^a	12 ^a	24 ^a	36 ^a			
Reference ^a	Leu	210±5	180±18	195±15	240±2	181±8	215±6	221±28	0.0079	0.0001	0.1296
10% ACCM ^a		248±33	155±2	175±27	196±15	217±5	215±2	217±17			
30% ACCM ^b		187±24	133±1	180±7	174±6	205±1	163±4	203±2			
30% WCM ^{ab}		197±6	158±1	185±7	168±19	204±6	225±2	229±39			
		0	3	6	9	12	24	36			
Reference ^b	Lys	1617±149	1520±160	1495±196	2081±46	1890±175	1589±127	1895±206	<0.0001	0.1612	0.0706
10% ACCM ^b		2570±641	1888±157	1204±29	1351±364	1468±477	1865±9	1884±209			
30% ACCM ^b		1986±158	1301±56	1677±378	1942±64	1806±187	1581±18	1787±75			
30% WCM ^a		2204±30	2387±137	2178±224	2252±259	2484±46	2780±4	2234±174			
		0 ^{ab}	3 ^{ab}	6 ^a	9 ^a	12 ^a	24 ^b	36 ^b			
Reference ^b	Met	89±1	92±15	112±9	117±9	103±8	95±13	77±8	0.0002	0.0003	0.6612
10% ACCM ^a		115±25	98±4	117±13	128±24	131±2	106±6	111±1			
30% ACCM ^a		125±9	116±9	135±8	142±9	139±8	101±1	97±4			
30% WCM ^a		101±3	120±6	143±3	124±8	134±0	115±4	116±3			
		0 ^{abc}	3 ^{bc}	6 ^{abc}	9 ^a	12 ^{ab}	24 ^c	36 ^{abc}			
Reference ^{ab}	Phe	116±1 ^b	124±14 ^b	149±9 ^{ab}	164±17 ^{ab}	129±4 ^{ab}	118±2 ^b	113±7 ^b	0.0062	0.0018	0.0100
10% ACCM ^a		134±38 ^{ab}	121±13 ^b	139±15 ^{ab}	154±3 ^{ab}	151±6 ^{ab}	119±6 ^b	198±2 ^a			
30% ACCM ^b		142±20 ^{ab}	113±9 ^b	106±28 ^b	160±0 ^{ab}	143±8 ^{ab}	99±2 ^b	114±8 ^b			
30% WCM ^b		105±6 ^b	122±7 ^b	147±1 ^{ab}	126±4 ^b	136±7 ^{ab}	111±1 ^b	108±6 ^b			

Table. 8.4. Continued

Diet	EAA	Sampling time (hours)							p-values		
		0	3	6	9	12	24	36	Diet	Time	Interaction
Reference	Thr	623±12	928±284	799±64	954±45	805±31	718±120	798±20	0.6015	0.6296	0.0593
10% ACCM		875±153	792±77	670±69	807±122	926±161	752±54	795±29			
30% ACCM		945±113	626±76	775±21	835±8	742±1	572±34	794±15			
30% WCM		531±63	770±35	820±30	701±6	791±5	870±1	842±55			
		0	3	6	9	12	24	36			
Reference	Trp	48±1	0±0	44±1	46±2	48±1	48±2	44±0	0.2222	-	-
10% ACCM		50±29	0±0	32±12	44±1	52±2	47±1	0±0			
30% ACCM		65±17	10±10	62±16	53±1	51±2	45±1	47±2			
30% WCM		46±0	44±2	47±4	45±0	53±2	42±0	45±1			
		0^a	3^c	6^{bc}	9^{bc}	12^b	24^a	36^a			
Reference ^b	Val	223±1	147±15	129±17	176±1	139±13	225±15	241±32	0.0042	<0.0001	0.0735
10% ACCM ^b		279±48	134±1	116±24	149±20	176±15	221±5	226±23			
30% ACCM ^b		246±12	129±0	167±10	165±17	210±1	197±9	226±1			
30% WCM ^a		229±11	164±4	202±2	180±19	212±7	271±7	259±34			
	CEAA	0	3	6	9	12	24	36			
Reference	Cys	0±0	0±0	0±0	0±0	0±0	0±0	0±0	-	-	
10% ACCM		0±0	0±0	0±0	5±2	0±0	0±0	0±0			
30% ACCM		0±0	0±0	0±0	0±0	0±0	0±0	0±0			
30% WCM		0±0	0±0	0±0	0±0	0±0	0±0	0±0			
		0^{ab}	3^b	6^{ab}	9^a	12^{ab}	24^{ab}	36^{ab}			
Reference ^a	Tyr	102±5	105±26	131±21	170±5	118±4	118±16	108±15	0.0066	0.0240	0.1753
10% ACCM ^a		134±45	93±11	95±0	141±20	125±4	117±22	173±5			
30% ACCM ^b		125±18	79±11	103±22	110±8	108±2	84±4	93±2			
30% WCM ^{ab}		100±0	94±9	115±1	107±6	112±1	96±4	103±4			

^aDiets formulated to contain 42% crude protein and 12% crude lipid. All diets contained 10% fish meal (FM) and 0% poultry-by-product meal. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC.

Table 8.5. Effects of including 0 (ref), 10 (10% ACCM) and 30% (30% ACCM) aerobically converted (ACCM) or 30% washed (30% WCM) carinata meals in diets on ratios of free essential amino acids (EAA) to lysine in the muscle of Hybrid Striped Bass at various sampling times (hours)^a

Diet ^a	EAA	Muscle Ratio ^b	Sampling times (hours)						
			0	3	6	9	12	24	36
Reference	Arg	1.19 ^c	0.04	0.04	0.03	0.04	0.06	0.05	0.06
10% ACCM			0.08	0.04	0.04	0.03	0.04	0.04	0.10
30% ACCM			0.05	0.07	0.06	0.04	0.05	0.05	0.06
30% WCM			0.03	0.04	0.04	0.03	0.05	0.05	0.04
			0	3	6	9	12	24	36
Reference	His	0.38 ^c	1.55	1.90	2.39	1.60	1.47	1.93	1.77
10% ACCM			1.02	1.56	3.13	2.78	2.73	1.96	1.59
30% ACCM			2.44	4.49	3.34	2.89	3.57	3.41	2.81
30% WCM			1.92	1.75	2.06	1.83	1.80	1.41	1.82
			0	3	6	9	12	24	36
Reference	Ile	0.54 ^c	0.14	0.06	0.06	0.07	0.05	0.12	0.12
10% ACCM			0.10	0.04	0.08	0.07	0.09	0.11	0.10
30% ACCM			0.15	0.06	0.09	0.07	0.10	0.10	0.12
30% WCM			0.09	0.05	0.07	0.06	0.07	0.09	0.11
			0	3	6	9	12	24	36
Reference	Leu	0.87 ^c	0.15	0.14	0.15	0.13	0.11	0.16	0.13
10% ACCM			0.11	0.09	0.17	0.17	0.17	0.13	0.13
30% ACCM			0.11	0.12	0.12	0.10	0.13	0.12	0.13
30% WCM			0.10	0.08	0.10	0.09	0.09	0.09	0.12
			0	3	6	9	12	24	36
Reference	Met	0.38 ^c	0.14	0.16	0.20	0.15	0.14	0.16	0.11
10% ACCM			0.12	0.14	0.26	0.25	0.23	0.15	0.15
30% ACCM			0.17	0.23	0.21	0.19	0.20	0.17	0.14
30% WCM			0.12	0.13	0.17	0.14	0.14	0.11	0.14
			0	3	6	9	12	24	36
Reference	Phe	0.49 ^c	0.15	0.17	0.20	0.16	0.14	0.15	0.12
10% ACCM			0.11	0.13	0.24	0.23	0.21	0.13	0.22
30% ACCM			0.15	0.18	0.13	0.17	0.16	0.13	0.13
30% WCM			0.10	0.11	0.14	0.12	0.11	0.08	0.10
			0	3	6	9	12	24	36
Reference	Thr	0.67 ^c	0.57	0.91	0.80	0.68	0.63	0.67	0.63
10% ACCM			0.51	0.63	0.83	0.89	0.94	0.60	0.63
30% ACCM			0.71	0.72	0.69	0.64	0.61	0.54	0.66
30% WCM			0.36	0.48	0.56	0.46	0.47	0.47	0.56
			0	3	6	9	12	24	36
Reference	Trp	0.12 ^d	0.25	0.00	0.25	0.18	0.21	0.25	0.19
10% ACCM			0.16	0.00	0.22	0.27	0.30	0.21	0.00
30% ACCM			0.27	0.06	0.31	0.23	0.24	0.24	0.22
30% WCM			0.17	0.15	0.18	0.17	0.18	0.13	0.17
			0	3	6	9	12	24	36
Reference	Val	0.61 ^c	0.23	0.16	0.14	0.14	0.12	0.23	0.21
10% ACCM			0.18	0.12	0.16	0.18	0.20	0.20	0.20
30% ACCM			0.20	0.16	0.16	0.14	0.19	0.21	0.21
30% WCM			0.17	0.11	0.15	0.13	0.14	0.16	0.19

^aDiets formulated to contain 42% crude protein and 12% crude lipid. All diets contained 10% fish meal (FM) and 0% poultry by-product meal. ACCM was hexane extracted (HE) carinata meal that was aerobically converted (AC) using fungi spp and single washed. WCM was HE carinata meal that was double washed with water without AC;

^bRatios of EAA to lysine in fish muscle were used to calculate the optimal concentrations of EAAs for muscle synthesis relative to lysine at each sampling time. Actual concentrations of EAAs at each sampling time were then divided by the calculated optimal concentrations for muscle synthesis to obtain a ratio of each EAA relative to requirement for each sampling time; ^cRawles et al. (2012); ^dUSDA (1987).

Figure 8.1. Temporal patterns of total essential amino acids in serum and dorsal white muscle in Hybrid Striped Bass fed diets containing 0 (ref), 10 (10 %ACCM) and 30% (30% ACCM) aerobically converted (ACCM) or 30% washed (30% WCM) carinata meals.

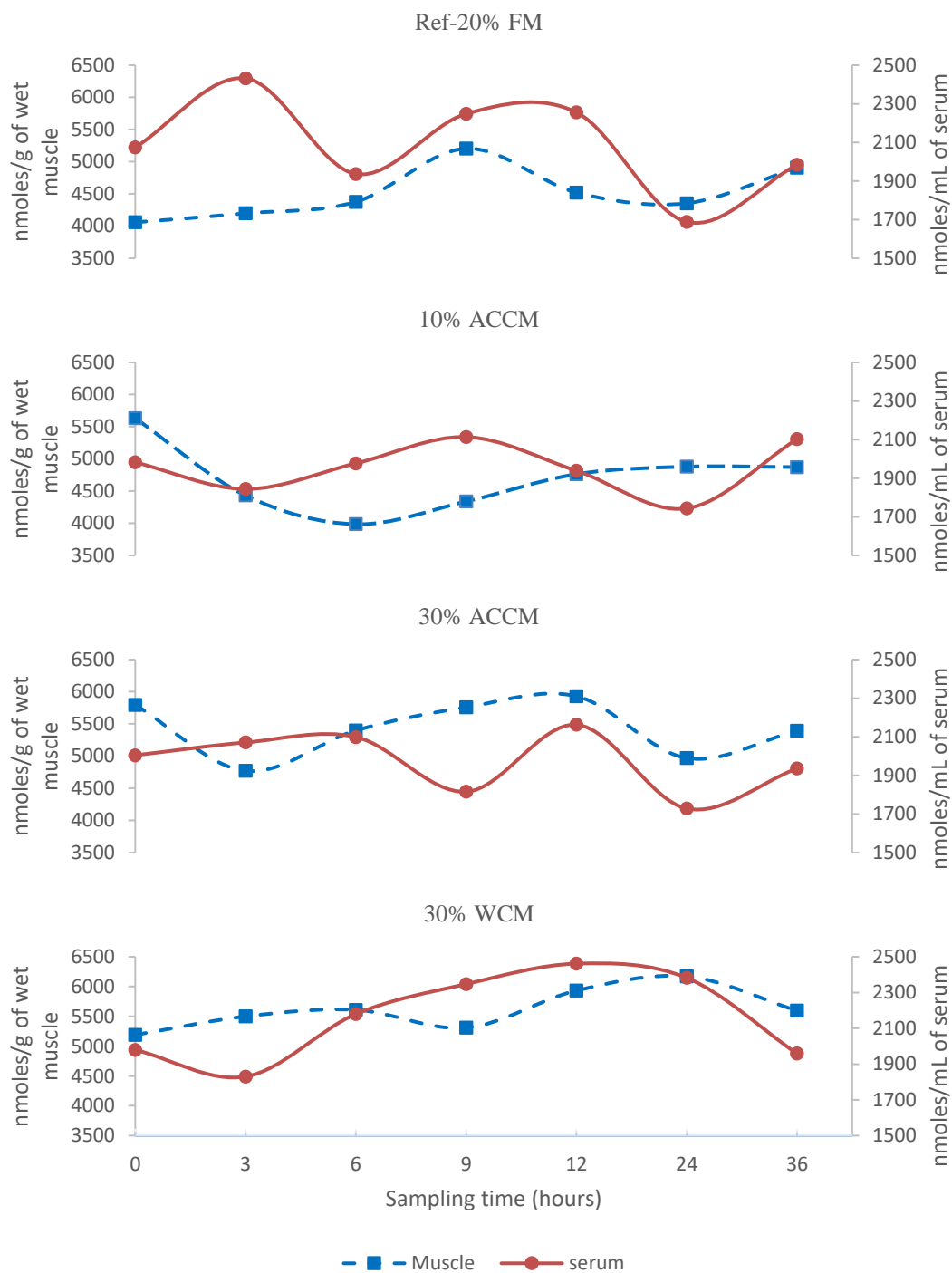


Figure 8.2. Effects of including 0 (ref), 10 (10 %ACCM) and 30% (30% ACCM) aerobically converted (ACCM) or 30% washed (30% WCM) carinata meals in Hybrid Striped Bass diets containing similar fish meal (FM) on cumulative serum free total essential amino acids (FEAA, nmoles mL⁻¹).

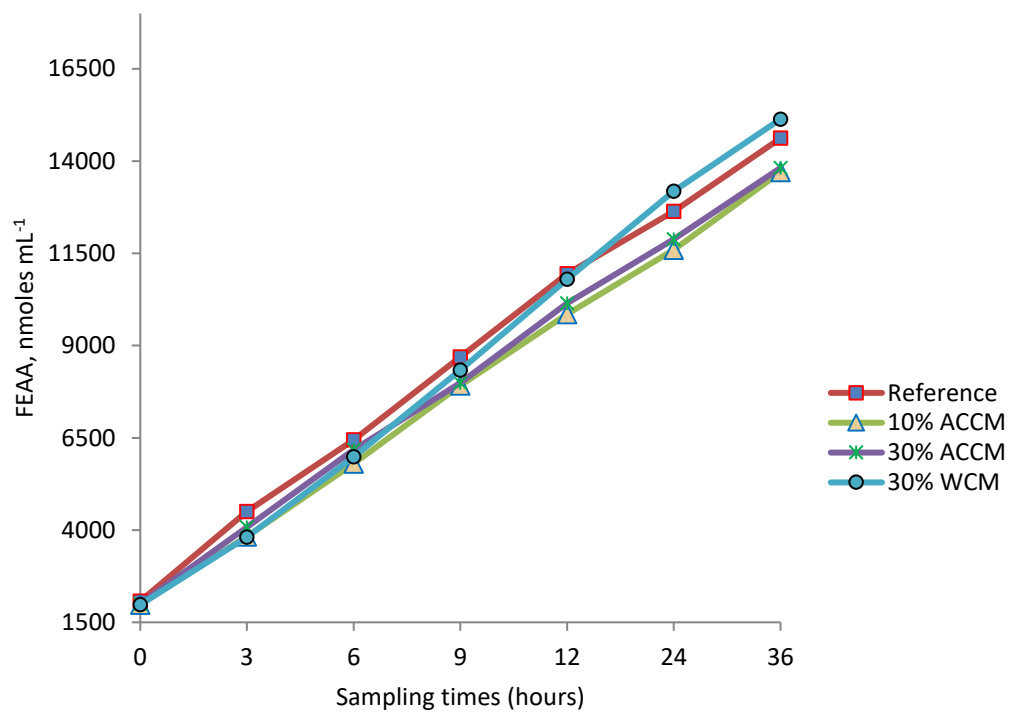
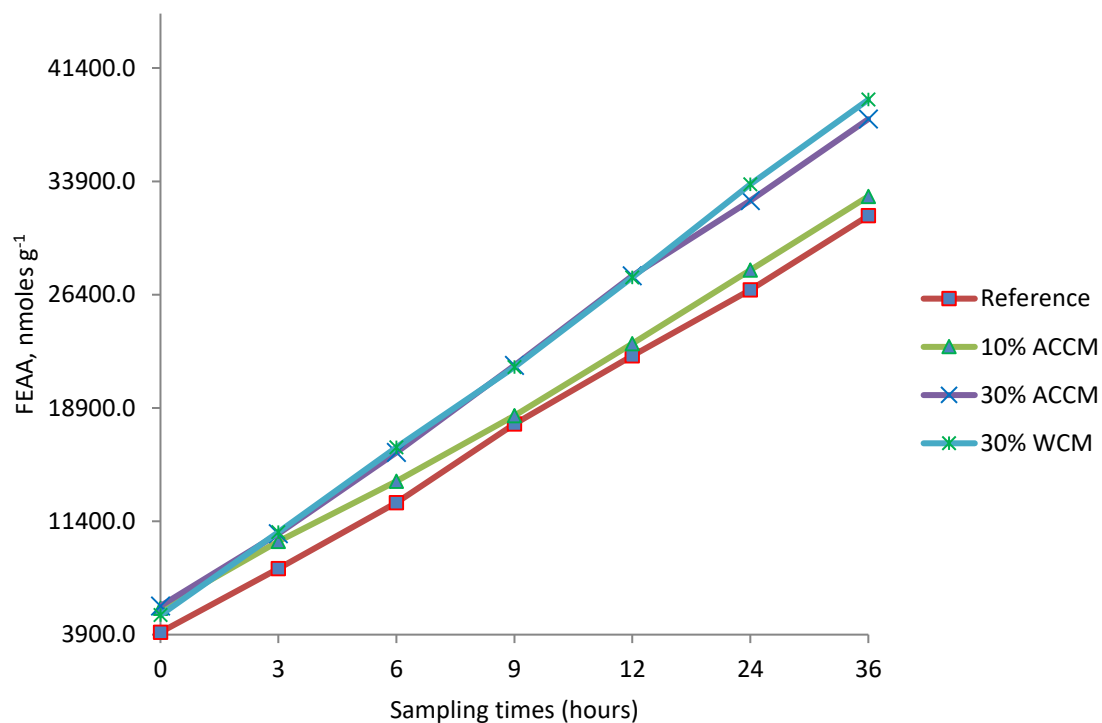


Figure 8.3. Effects of including 0 (ref), 10 (10 %ACCM) and 30% (30% ACCM) aerobically converted (ACCM) or 30% washed (30% WCM) carinata meals in Hybrid Striped Bass diets containing similar fish meal (FM) on cumulative muscle free total essential amino acids (FEAA, nmoles g⁻¹).



CHAPTER 9

Conclusion and Research Needs

Traditionally, carinata and camelina seed meals have been included in animal feeds at $\leq 10\%$ of diets mainly due to high concentrations of glucosinolates (GLS) and sinapine. Recent fish nutrition studies have focused more on camelina than carinata meals possibly because of the generally lower GLS and sinapine concentrations in camelina seeds. Carinata and camelina seeds were processed by cold press (CP) followed by extrusion (EX), solvent extraction (SE) and aerobic conversion (AC) or a process combination to determine process(es) yielded meals of high nutritional value to fish. Carinata meals generally yielded more crude protein and lower fiber. Most GLS were reduced ($>70\%$) by AC in both meal types. However, AC increased crude fiber in all meals, thus reducing their digestibility. Palatability in Rainbow Trout *Oncorhynchus mykiss* (RBT) was generally improved by SE and AC but was not different by meal type. Apparent digestibility coefficients (ADCs) for protein were generally higher in carinata than camelina meals for both RBT and Hybrid Striped (Sunshine) Bass *Morone chrysops* ♀ x *M. saxatilis* ♂ (HSB).

Based on meal composition, carinata is a good source of protein for fish feeds because the processed meals are high ($>45\%$) in crude protein that is highly digestible. Traditionally, de-oiled carinata meal fed to animals is not processed beyond oil extraction to reduce GLS and sinapine, which makes the meal unpalatable and thus the preference for lower GLS and sinapine containing de-oiled seed meal types such as camelina meal. However, reduction of GLS in carinata meals only requires addition of water to the meal to enhance GLS hydrolysis; allyl isothiocyanates formed by GLS hydrolysis are volatile

and depleted during aqueous mixing and drying of the meal. Continuous mixing of the meal in water enhances GLS breakdown and the centrate discarded contains sinapine, soluble sugars, and other soluble antinutrients. Therefore, any meal processing involving a washing step improves overall meal palatability. However, AC has not been effective at reducing the crude fiber content of the meal to improve nutrient utilization in fish. Fish nutritionist mainly analyze crude fiber (cellulose and insoluble hemicellulose and lignin) as part of the routine proximate composition analyses of feed ingredients and diets. However, plant meals contain other nonstarch polysaccharides such as pectins and gums, soluble hemicellulose and lignin, and β glucans that may all be detrimental to utilization of nutrients in plant meals.

Research is underway to use food grade exogenous enzymes to lower crude fiber in the AC meal. Preliminary studies with RonozymeVP (cocktail of β -Glucanase, hemicellulase and pectinase) resulted in a reduction of crude fiber to 5.8%. However, the activities of individual enzyme in RonozymeVP were low. In addition, RonozymeVP was not readily soluble in water. Testing the individual performance of more soluble carbohydrases from an alternative source indicates higher enzyme activity. Cellulase alone reduced the crude fiber to 7.7%, and resulted in an increase in crude protein to 65%. After evaluating the efficacy of other individual soluble carbohydrases such as pectinase, hemicellulase, glucanase, etc., the desired combination can be tested as a cocktail or in a stepwise manner, with a target of reducing crude fiber to $\leq 3\%$ of the meal and providing $\geq 70\%$ protein on a as is basis.

The GLS (22-62 μ moles) and sinapine (4.3-6.1 mg) concentrations per gram of de-oiled carinata meal are high and variable among carinata strains. However, the

combined tolerances of HSB to GLS and sinapine from carinata meal were ≤ 2.65 μ moles and ≤ 0.31 mg/g of diet, respectively. Considering the 50% reduction in GLS and sinapine during feed extrusion, the GLS and sinapine tolerance limits above should act as a guide to estimate the maximum inclusion of processed carinata meals containing various GLS and sinapine concentrations in fish diets. Alternatively, the maximum GLS and sinapine concentrations can be set as targets not to be exceeded in processed meals, based on the desired inclusion level of processed carinata meal in fish diets. These recommendations will be more helpful in producing feeds where carinata meals are not processed beyond oil extraction.

The overall performance of RBT fed 15% aerobically converted carinata meal (ACCM) was good and only 5% fish meal (FM) as the sole animal protein showed that processed carinata can support good fish growth. However, the reduction of RBT growth fed diets containing $\leq 10\%$ FM (or animal protein) showed that higher replacements of FM in RBT diets may occur if some of the less expensive animal by-product meals such as blood meal, poultry by-product meal, hydrolyzed feather, etc. are added to diet formulations. Therefore, additional studies should consider replacing most of the FM component ($\sim 5\%$) with processed carinata, but in combination with other more economical replacement animal by-products in RBT diets containing higher ($\sim 40\%$) animal protein.

Similar performance of HSB fed diets containing up to 30% ACCM and similar animal meals to the reference diet showed that high inclusions of processed carinata meal could be included in fish diets if they contain adequate animal meals. Additionally, better feed utilization of double washed carinata without AC (WCM) included at 30% of the

diet suggested better nutrient utilization, even with a slightly higher crude fiber content. Carinata contains high a high iron content that likely increases the Hk and Hb content of fish fed carinata meals. However, the iron content is decreased by meal washing, with the second wash likely decreasing more iron in the meal, resulting in less Hk and Hb. The associated reduction in lysozyme with increasing GLS in diets may not be observed with meals subjected to AC, because GLS concentrations in the meals are greatly reduced before further reduction by diet manufacture. Future processing of carinata meals should likely involve at least two wash steps. Currently, it is not clear why the second wash improved nutrient utilization, therefore more research is needed to determine what soluble compound is thoroughly removed with the second wash, resulting in better nutrient utilization.

Followup studies showed that high (30%) inclusions of ACCM but not WCM lowered protein ADCs, which reaffirms the need to determine the antinutrient in ACCM that was not thoroughly removed by a single wash. Amino acids from plant meals are released at a much slower rate than amino acids from animal meals, which explains the lower total essential amino acids (EAAs) in serum of RBT fed diets in which FM was replaced with ACCM. Although, HSB diets contained similar amounts of animal meals and the lower protein ADC for ACCM resulted in lower total EAAs in serum; the higher protein ADC of WCM did not affect total EAAs in serum. Better utilization of WCM nutrients reaffirms the need to have at least two wash steps during carinata processing.

Ratios of each EAA to lysine in muscles showed which amino acids were limiting for muscle synthesis at a given lysine concentration over time. This approach provides a better method of determining limiting amino acids for protein synthesis than just

considering which EAAs are at the lowest concentration, the most common approach.

The EAA:lysine ratios provided a better explanation of protein utilization in RBT than for HSB. Therefore, feed formulations, especially for those diets low in FM/animal meal content, should emphasize increasing the concentrations of EAAs that are low in plant meals such as lysine and methionine to meet the dietary EAA requirements.

Combinations of FM and / or animal meals with plant meals that result in optimal free muscle EAA:lysine ratios for muscle synthesis should be evaluated because most protein accretion in fish occurs in muscle.